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PRINCIPAL INVESTIGATOR: Selvarangan Ponnazhagan, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, Alabama 35294-0109

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Selvarangan Ponnazhagan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Alabama at Birmingham
Birmingham, Alabama 35294-0109

E-Mail: sponnazh@path.uab.edu

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13. ABSTRACT (Maximum 200 Words)

From the studies performed during the last one year, we determined the effects of AAV-mediated anti-angiogenic gene therapy as a combination therapy to chemotherapy. Based on the kinetics of rAAV transgene expression and the requirement of second-strand synthesis as a rate-limiting step, we continued our studies in a prevention model. Also we continued to use the cell line SKOV3.ip1 instead of human breast cancer cell lines to avoid time delay due to slower growth kinetics *in vivo*. rAAV-encoding angiostatin and endostatin was injected prior to tumor cell implantation. Three weeks following vector administration, 10^6 SKOV3.ip1 cells were implanted. Cohorts of mice also received no vector treatment, rAAV-GFP (non-specific vector control), chemotherapy alone, or a combination of rAAV endostatin+angiostatin and chemotherapy. Chemotherapy was administered during the first week after tumor implantation by intraperitoneal injection of 20 mg/kg taxol. Based on the results of these studies, we inferred the potential of AAV-mediated anti-angiogenic gene therapy in combination with chemotherapy. In the next year, we will determine whether such a combination therapy would provide regression of established tumors.

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INTRODUCTION

The mortality associated with breast cancer is primarily due to systemic dissemination of the disease to which conventional therapies such as surgery, radiation therapy and chemotherapy fail to provide long-term cure. Thus, development of novel approaches is important for the treatment of metastatic breast disease. Among the possible targets, the tumor endothelium is more promising since the endothelial cell growth, termed angiogenesis, is a crucial event for tumor growth and metastasis (1-3). Earlier studies using purified anti-angiogenic factors indicated a need for constant administration, which involves complexities in production, and possible side effects. To overcome these limitations, more recently, approaches based on the introduction of anti-angiogenic genes using plasmid DNA, adenoviral, and retroviral vectors are being attempted. However, these vectors have limitations such as inefficient long-term persistence, host immunity and a requirement of active cell division respectively.

Recombinant adeno-associated virus (rAAV) vectors, on the other hand, are unique group of DNA containing viruses, which can transduce both dividing and non-dividing cells. rAAV vectors are less immunogenic and establish long-term persistence in host cells, hence, possess the advantage for sustained long-term expression of the transgenes. Based on these advantages, we hypothesized that long-term gene therapy for breast cancer by AAV-mediated sustained expression of anti-angiogenic factors *in vivo* will be efficacious both as a primary therapy for established tumors and as an adjuvant therapy for the recurrence of radiation- and chemo-resistant tumors. Further, we hypothesized that development of breast cancer-specific rAAV containing anti-angiogenic genes would not only increase targeted-transduction but also minimize the vector dose and associated toxicity, if any. The proposed specific aims of the project are:

1) To determine the anti-angiogenic potential of rAAV encoding angiostatin, endostatin and soluble VEGF receptor in biologically relevant assays.

2) To evaluate the *in vivo* efficacy of rAAV encoding the anti-angiogenic factors in inhibiting breast cancer growth and metastasis in a murine model.

3) To determine the efficacy of anti-angiogenic gene therapy as an adjuvant therapy for recurrence of radiation resistant breast cancer growth and metastasis in a murine model *in vivo*.

BODY

In the progress report for 2002-2003, we reported research accomplishments on

a) Inhibition of endothelial cell proliferation by rAAV vectors encoding, angiostatin, endostatin, endostatin+angiostatin or sFlt-1, and
b) Stable rAAV-mediated expression of human sFlt-1, endostatin, angiostatin or endostatin+angiostatin confers significant tumor protection in an *in vivo* murine xenograft model.

Further to this study, in the last one year, we determined the effects of recombinant adeno-associated virus (rAAV)-mediated anti-angiogenic gene therapy as a combination therapy to chemotherapy. In our previous studies, we determined that a rAAV containing both endostatin and angiostatin genes from a bicistronic expression cassette provided maximum therapeutic effect in abrogating the growth of an angiogenesis-dependent human epithelial cancer cell line, SKOV3.ip1, grown as a subcutaneous xenograft. Hence, in studies to determine the synergistic effects of anti-angiuogenic gene therapy with chemotherapy, we used only the rAAV encoding both angiostatin and endostatin. Based on the kinetics of rAAV transgene expression and the requirement of second-strand synthesis as a rate-limiting step, we continued our studies in a prevention model. Also we continued to use the cell line SKOV3.ip1 instead of human breast cancer cell lines to avoid time delay due to slower growth kinetics *in vivo*. As in the model we recently established, rAAV-encoding angiostatin and endostatin was injected prior to tumor cell implantation. Three weeks following vector administration, 10^6 SKOV3.ip1 cells were implanted intraperitoneally. Cohorts of mice also received no vector treatment, rAAV-GFP (non-specific vector control), chemotherapy alone, or a combination of rAAV endostatin+angiostatin and chemotherapy. Chemotherapy was administered during the first week after tumor implantation by intraperitoneal injection of 20 mg/kg taxol, two times (days 4 and 7 after tumor implantation). Results, shown in Figures 1 and 2 demonstrate the effectiveness of this therapy on both tumor-free survival and on reducing the proliferation of tumor cells in mice, which showed tumor growth. In the next year, we will determine the effects of rAAV-endostatin+angiostatin therapy either as an individual therapy or as a combination therapy to chemotherapy in a therapeutic model of breast cancer. The slow growth kinetics of human breast cancer cells as xenografts will be more suited for this experiment.

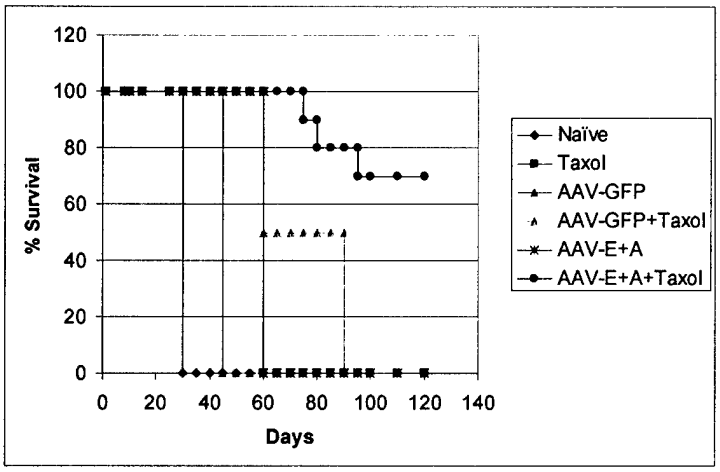
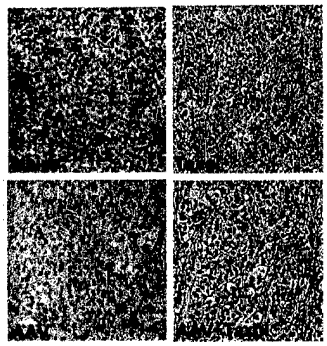


Figure 1. Survival of mice following administration of rAAV ± chemotherapy (E+A) and subsequent tumor challenge. A dose of $3x10^{11}$ particles of rAAV encoding GFP, or endostatin+angiostatin or sFlt1 was injected intraperitoneally into athymic nude mice. Three weeks after the vector injection, the animals were challenged with 10^7 SKOV3.ip1 cells in each flank subcutaneously. Taxol injections were also given intraperitoneally at a concentration of 20 mg/kg, two times (days 4 and 7 after tumor implantation). The growth of tumors was measured twice weekly.

Figure 2. Immunohistochemical analysis of tumor cells for proliferation index. Following no treatment (naive), or treatment with taxol, rAAV encoding endostatin+angiostatin (AAV) or a combination of these two (AAV+Taxol), tumor tissue was removed from mice during sacrifice, fixed in buffered-formalin and embedded in paraffin. The tumor cell proliferation was determined by staining 5 μm sections of the fixed tissue with Ki67 antibody. Magnification x20.



KEY RESEARCH ACCOMPLISHMENTS

1. Established that rAAV-mediated gene therapy with anti-angiogenic factors angiostatin, endostatin provided significant anti-tumor effect in combination with the chemotherapeutic drug Taxol.
2. Tumor cells treated with the combination of rAAV endostatin+angiostatin showed markedly less proliferation rate compared to untreated control.

REPORTABLE OUTCOMES

(Papers published or communicated)

1. Ponnazhagan, S. Mahendra, G., Kumar, S., Shaw, D., Meleth, S., Stockardt, R., and Grizzle, W.E. Adeno-associated virus-2-mediated anti-angiogenic gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. *Cancer Res.* 64, 1781-1787, 2004.

2. Mahendra, G., Isayeve, T., Mahasreshti, P., Curiel, D.T., Stockardt, R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., and Ponnazhagan, S. Anti-angiogenic gene therapy through adeno-associated virus 2-mediated stable expression of soluble Flt-1 receptor *Cancer Gene Ther.* 2004 (in press).

3. Isayeva, T., and Ponnazhagan, S. Anti-angiogenic gene therapy for cancer. *Int. J. Oncol.* 2004 (in press)

4. Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2-mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer. Cancer Res. 2004 (communicated)

(Results presented in conferences)

1. Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D., Stockard, C.E., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated gene therapy: long-term efficacy of a combination vector over individual therapy. 6th Annual Meeting of the American Society for Gene Therapy, Washington D.C. 2003.

2. Ponnazhagan, S., Mahendra, G., Kumar, S., Shaw, D.R., Stockard, C.R., Grizzle, W.E., and Meleth, S. Adeno-associated virus 2-mediated anti-angiogenic cancer gene therapy: long-

term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. 95th Annual Meeting of the American Society for Cancer Research, Orlando, FL, 2004.

3. Chaudhuri, T.R., Cao, Z., Ponnazhagan, S., Stargel, A., Simhadri, P.L., Zhou, T., LoBuglio, A.F., Buchsbaum, D.J., and Zinn, K. Detection of disseminated breast cancer growth and treatment response using non-invasive bioluminescence imaging (BI). 40th Annual meeting of the American Society of Clinical Oncology, New Orleans, LA, June 2004.

4. Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2 -mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer. 7th Annual meeting of the American Society of Gene Therapy, Minneapolis, MN, June 2004.

CONCLUSIONS

From the work done in the last year, we inferred the potential of AAV- mediated anti-angiogenic gene therapy in combination with chemotherapy. These studies were done in a preventive model of epithelial cancer. In the next year, we will determine whether, such a combination therapy would provide regression of established tumors.

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2. Folkman , J., Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., 1971,285:1182-6.
3. Folkman, J. Anti-angiogenic gene therapy. Proc. Natl. Acad. Sci. U.S.A. 1998,95:9064-9066.

PERSONNEL RECEIVING PAY FROM THIS GRANT

Selvarangan Ponnazhagan, Ph.D.

APPENDICES

Copies of the following manuscripts enclosed:

1. Ponnazhagan, S. Mahendra, G., Kumar, S., Shaw, D., Meleth, S., Stockardt, R., and Grizzle, W.E. Adeno-associated virus-2-mediated anti-angiogenic gene therapy: long-term efficacy of a vector encoding angiostatin and endostatin over vectors encoding a single factor. *Cancer Res.* 64, 1781-1787, 2004.

2. Mahendra, G., Isayeva, T. Mahasreshti, P., Curiel, D.T., Stockardt, R., Grizzle, W.E., Alapati, V., Singh, R., Siegal, G.P., and Ponnazhagan, S. Anti-angiogenic gene therapy through adeno-associated virus 2-mediated stable expression of soluble Flt-1 receptor *Cancer Gene Ther.* 2004 (in press).

3. Isayeva, T., and Ponnazhagan, S. Anti-angiogenic gene therapy for cancer. *Int. J. Oncol.* 2004 (in press)

4. Isayeva, T., Ren, C., and Ponnazhagan, S. Recombinant adeno-associated virus 2-mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer. Cancer Res. 2004 (communicated)

[CANCER RESEARCH 64, 1781–1787, March 1, 2004]

Appendix 1

Adeno-Associated Virus 2-Mediated Antiangiogenic Cancer Gene Therapy: Long-Term Efficacy of a Vector Encoding Angiostatin and Endostatin over Vectors Encoding a Single Factor

Selvarangan Ponnazhagan,[1] Gandham Mahendra,[1] Sanjay Kumar,[1] Denise R. Shaw,[2] Cecil R. Stockard,[1] William E. Grizzle,[1] and Sreelatha Meleth[2]

Departments of [1]Pathology and [2]Medicine and the Comprehensive Cancer Center, The University of Alabama at Birmingham, Birmingham, Alabama

ABSTRACT

Angiogenesis is characteristic of solid tumor growth and a surrogate marker for metastasis in many human cancers. Inhibition of tumor angiogenesis using antiangiogenic drugs and gene transfer approaches has suggested the potential of this form of therapy in controlling tumor growth. However, for long-term tumor-free survival by antiangiogenic therapy, the factors controlling tumor neovasculature need to be systemically maintained at stable therapeutic levels. Here we show sustained expression of the antiangiogenic factors angiostatin and endostatin as secretory proteins by recombinant adeno-associated virus 2 (rAAV)-mediated gene transfer. Both vectors provided significant protective efficacy in a mouse tumor xenograft model. Stable transgene persistence and systemic levels of both angiostatin and endostatin were confirmed by *in situ* hybridization of the vector-injected tissues and by serum ELISA measurements, respectively. Whereas treatment with rAAV containing either endostatin or angiostatin alone resulted in moderate to significant protection, the combination of endostatin and angiostatin gene transfer from a single vector resulted in a complete protection. These data suggest that AAV-mediated long-term expression of both endostatin and angiostatin may have clinical utility against recurrence of cancers after primary therapies and may represent rational adjuvant therapies in combination with radiation or chemotherapy.

INTRODUCTION

Increasing evidence demonstrates the importance of angiogenesis in solid tumor growth and metastasis (1–4). In the absence of neovasculature, tumors do not grow beyond a few millimeters and remain dormant (5, 6). Thus, novel antiangiogenic treatment strategies that can effectively control tumor growth are under intense investigation. Although many antiangiogenic factors have been implicated in the regulation of tumor growth and metastasis, the most potent have been angiostatin, endostatin, thrombospondin-1, tissue inhibitor of metalloproteases, and soluble vascular endothelial growth factor (VEGF) receptors (7–10).

Preclinical studies using purified antiangiogenic factors indicated therapeutic effects of antiangiogenic compounds in minimizing the size of established tumors (11–15). However, clinical trials with some of these factors have not demonstrated expected antitumor effects (16–19). Administration of purified antiangiogenic factors, although capable of producing significant growth inhibition of tumor cells in animal models, may be limited by their short half-life. Hence, production of antiangiogenic factors after gene transfer may overcome these limitations.

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Requests for reprints: Selvarangan Ponnazhagan, Department of Pathology, LHRB 513, 701 19th Street South, University of Alabama at Birmingham, Birmingham, AL 35294-0007. Phone: (205) 934-6731, Fax: (205) 975-9927, E-mail: sponnazh@path.uab.edu.

The potential of antiangiogenic gene therapy in cancer is currently being evaluated using viral and nonviral vectors (20–23). In contrast to genetic therapies targeting tumor cells directly with genes encoding prodrug-converting enzymes or cytokines/chemokines for oncolysis, which requires high-efficiency transduction of recombinant vectors to cancer cells directly, antiangiogenic gene therapy requires vectors capable of sustained, long-term expression without vector-associated toxicity or immunity. Additionally, systemic levels of antiangiogenic factors by gene transfer may be accomplished by targeting nontumor cells, using normal tissues to provide a stable platform for transgene expression as secretory proteins. Adeno-associated virus (AAV)-based vectors are nonpathogenic and less immunogenic compared with other gene therapy vectors. The AAV genome persists stably in transduced cells and affects long-term transgene expression. Thus, AAV meets the requirements for gene transfer vectors that may be used for antiangiogenic therapy.

The present study evaluated recombinant AAV (rAAV) encoding secretable forms of human angiostatin and endostatin. The results demonstrate a strong antiproliferative effect of rAAV-mediated angiostatin or endostatin gene transfer on primary human umbilical vein endothelial cells (HUVEC) *in vitro* and significant protective effect against the growth of a human angiogenesis-dependent tumor xenograft *in vivo*. Furthermore, the combination of both angiostatin and endostatin long-term gene therapy from a single vector resulted in a synergistic effect over therapy with vectors encoding a single factor alone.

MATERIALS AND METHODS

Cells and Reagents. Human embryonic kidney cell line 293 was purchased from American Type Culture Collection and maintained in Iscove's modified essential medium supplemented with 10% newborn calf serum. Human ovarian cancer cell line SKOV3.ip1 was a kind gift of Dr. David Curiel (The University of Alabama at Birmingham, Birmingham, AL) and maintained as described previously (24). Primary HUVEC were a gift of Dr. Raj Singh (The University of Alabama at Birmingham, Birmingham, AL). Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corp. (Madison, WI). Mouse monoclonal (clone 79735) and goat polyclonal antibodies to human angiostatin were obtained from R&D Systems (Minneapolis, MN), and a mouse monoclonal antibody to human endostatin (clone EN2.1.99) was obtained from Leinco Technologies (St. Louis, MO). Secondary antibodies and colorimetric substrates were purchased from Amersham (Piscataway, NJ). Purified recombinant human angiostatin was purchased from R&D Systems.

Construction of Recombinant Plasmids, Production, and Purification of rAAV. All rAAV plasmids were constructed using pSub201 as the back bone (25). cDNA containing human angiostatin and endostatin sequences were isolated from a plasmid pBlast human Endo::Angio (Invivogen, San Diego, CA). For construction of the rAAV plasmid encoding endostatin, a region containing the human interleukin 2 secretory signal sequence was genetically fused to the endostatin coding region, amplified from the plasmid pBlast human Endo::Angio by PCR, and subcloned into an AAV plasmid containing cytomegalovirus (CMV) promoter, sequences of internal ribosome entry site (IRES), and a green fluorescent protein (GFP) gene followed by a synthetic polyadenylation signal sequence (polyA). Construction of rAAV encoding

human angiostatin was done in two steps. Initially, the coding region of human angiostatin was amplified by PCR from the plasmid pBlast human Endo::Angio and subcloned in pBluescript vector (Stratagene, La Jolla, CA) with a double-stranded oligomer (5′-TCGAGATGGAACATAAGGAAGTG-GTTCTTCTACTTCTTTTATTTCTGAAATCAGGTCAAG-3 and 5′-GATCCTTGACCTGATTTCAGAAATAAAAGAAGTAGAAGAACCA-CTTCCTTATGTTCCATC-3′) containing the plasminogen secretory signal sequence, in a three-way ligation. Subsequently, the region containing the secretory sequence and angiostatin gene was excised and subcloned downstream of CMV promoter in a rAAV plasmid as described for rAAV-endostatin construct.

A bicistronic rAAV plasmid containing both endostatin and angiostatin was constructed in three steps. First, the IRES and endostatin sequences were amplified by PCR and subcloned in pBluescript vector (Stratagene). Then, a region containing plasminogen secretory signal and angiostatin was isolated from the plasmid described above and subcloned upstream of IRES-endostatin cassette in pBluescript vector. Later, the portion containing plasminogen secretory sequence, angiostatin, IRES, and endostatin was isolated and subcloned in a rAAV vector containing CMV promoter and a synthetic polyA.

Packaging of all of the recombinant AAV plasmids was done in an adenovirus-free system as described previously (26). Purification of virions was done by discontinuous iodixanol gradient centrifugation followed by affinity purification on a heparin-agarose column (26). Particle titers of the purified virions were determined by quantitative slot blot analysis as described previously (27–29).

Western Blot Analysis. Western blot analysis was performed using conditioned media obtained from rAAV-transduced 293 cells. Briefly, conditioned media obtained after mock-transduction, transduction of AAV-endostatin, AAV-angiostatin, or AAV-endostatin plus AAV-angiostatin vectors was concentrated 5-fold, and 20 μl from each was electrophoretically separated on 10% SDS polyacrylamide gels (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride membranes and immunodetection performed using mouse monoclonal antibodies to either human angiostatin (clone 79735) or endostatin (clone EN2.1.99) as primary antibody and goat antimouse secondary antibody conjugated to horseradish peroxidase as secondary antibody. Detection of bands was by enhanced chemiluminescent substrate as previously described (30).

Endothelial Cell Proliferation Assay. Early passage HUVECs were seeded into gelatin-coated 96-well tissue culture plates at a density of 5×10^3 cells/well and grown in EGM-2 medium containing hydrocortisone, human fibroblast growth factor β, VEGF, ascorbic acid, heparin, human epidermal growth factor, and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). Twenty-four h later, 100 μl of fresh medium containing 1, 10, or 25 μl of conditioned medium from 293 cells transduced with 100 multiplicity of infection of rAAV encoding various trasgenes were added. As a positive control, purified recombinant human angiostatin was added at concentrations of 1, 10, or 25 ng/ml. Each condition was performed in triplicate. Seventy-two h later, cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay with a commercial kit (Promega), following the manufacturer's instructions. Proliferation index was expressed in percentage compared with HUVEC grown in the presence of complete medium without addition of any conditioned medium or recombinant angiostatin.

***In Vivo* Studies.** Six-week old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD). Maintenance of the animals was done following the guidelines of the Institutional Animal Care and Use Committee, and all experimental procedures were approved by the Institutional Animal Care and Use Committee and the Occupational Health and Safety Department of the University of Alabama at Birmingham. Particles (3×10^{11}) of rAAV encoding GFP, endostatin, angiostatin, or endostatin plus angiostatin, in normal saline, were injected in a volume of 100 μl into the quadriceps muscle of the hind limbs. Naïve animals did not receive any vector. Three weeks after vector administration, each mouse received implantation s.c. in two sites on bilateral flanks with 10^7 SKOV3.ip1 cells. Tumor size was measured at least twice every week with a digital caliper for two-dimensional longest axis (L in mm) and shortest axis (W in mm), and tumor volume calculated using the following formula: volume in $mm^3 = (L \times W^2)/2$. When tumor growth exceeded 1800 mm^3, animals were humanely euthanized. Surviving mice were sacrificed 130 days after tumor cell implantation and experiments were terminated. Blood samples were collected from all animals before vector administration, before tumor cell implantation, and at sacrifice, for ELISA measurements of serum angiostatin and endostatin levels. Regions of the quadriceps muscle of sham or vector injection were also isolated at the time of sacrifice for immunohistochemistry and *in situ* hybridization.

ELISA for Serum Angiostatin and Endostatin Levels. For the measurement of serum angiostatin, a sandwich ELISA was developed. Ninety-six-well ELISA plates were coated overnight at 4°C with a mouse antihuman angiostatin monoclonal antibody (clone 79735) at a concentration of 10 μg/ml in borate saline buffer (BS; pH 8.6). Next day, the antibody was discarded and wells blocked with 150 μl of BSA in BS (BS-BSA) for 45 min at room temperature. Serum samples, diluted 1:3 in BS-BSA were added to the wells and incubated overnight at 4°C. All of the samples were analyzed in triplicate. After washing five times with PBS containing 0.5% Tween 20, a polyclonal antihuman angiostatin antibody, biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin reagent (Pierce), was added at a concentration of 1 μg/ml in BS-BSA and incubated at room temperature for 5 h. The contents were then discarded and plates washed five times with PBS containing 0.5% Tween 20 after which streptavidin-conjugated alkaline phosphatase was added and incubated for 30 min at room temperature. Color development was done with the addition of pNPP chromogenic substrate (Sigma) and incubated at room temperature for 20 min. Absorbance at 405 nm was measured in an ELISA plate reader. As a reference standard, known concentrations of human recombinant angiostatin from 0 to 1000 ng/ml were included in triplicate. Serum endostatin levels were determined using a commercial ELISA kit (Cytimmune Sciences Inc, College Park, MD) following the manufacturer's protocol.

***In Situ* Hybridization.** A digoxigenin (DIG)-labeled DNA probe containing sequence of CMV promoter was generated by PCR using PCR-DIG labeling mix^{Plus} (Roche Molecular Diagnostics, Indianapolis, IN) following manufacturer's protocol. Formalin-fixed tissues were sectioned at 5-μm thickness, deparaffinized in xylene, and rehydrated through a series of graded-ethanol and PBS. Slides were then treated with 0.01 M citrate buffer (pH 6.0) at 42°C for 3 h. Prehybridization was performed at 65°C for 2 h in hybridization solution (ULTRAhyb, Ambion, TX). The hybridization reaction was carried out with approximately 400 ng/ml of DIG-labeled DNA probe at 65°C overnight. After thoroughly washing the excess probe, immunohistochemical detection of hybridization signals was performed using the DIG nucleic acid detection kit (Roche Molecular Diagnostics). Counterstaining of sections was done with diluted eosin solution for 1–2 min and slides mounted in Crystal/Mount (Biomeda, Forest City, CA).

Immunohistochemistry. Quadriceps muscles of mice were harvested and fixed immediately in alcoholic-formalin (PenFix; Richard-Allan, Kalamazoo, MI) for 18 h at room temperature. Tissues were dehydrated in graded alcohol and embedded in paraffin. Five-μm sections on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling for 10 min in 0.01 M citrate buffer (pH 6.0) in a microwave oven. All sections were pretreated with a 3% aqueous solution of H_2O_2 for 5 min to quench endogenous peroxidase. Sections were then treated with 3% goat serum for 1 h at room temperature to reduce nonspecific staining followed by 1-h incubation with monoclonal antibodies to either endostatin (clone EN2.1.99, 10 μg/ml) or angiostatin (clone 79735, 5 μg/ml). The remainder of staining procedure was performed using a Universal Mouse Kit (Biogenex, Sam Ramon, CA), which contained biotinylated goat antibody to mouse immunoglobulin and a horseradish peroxidase-streptavidin complex. Diaminobenzidine tetrahydrochloride was used as a substrate for the visualization of antigen-antibody complex. Slides were minimally counterstained with hematoxylin.

Statistical Analyses. Nonparametric Kuskall Wallis tests or Wilcoxon's rank-sum test were used to test for differences in cell proliferation between the groups, depending on the number of groups included in the test. The association for the presence or absence of tumor with each treatment condition was tested for statistical significance using χ^2 test. Next, the distribution of tumor volumes was examined and log transformed to fit a normal distribution. A general linear mixed model was fit to the data. The dependent variable was the logged tumor volume and the predictors were treatment, day after treatment, and an interaction between treatment and day. All three were statistically significant predictors. $P < 0.05$ was considered to indicate significant difference between data sets.

RESULTS

Generation of High-Titer rAAV Encoding Human Angiostatin and/or Endostatin. On the basis of the long-term expression capabilities of rAAV in transduced muscle tissue without deterioration of transgene-positive cells by host immune cells, we chose muscle as a secretory organ for angiostatin and endostatin transgene expression. We included signal peptide sequences upstream of both angiostatin and endostatin genes. The human interleukin 2 secretory signal was included upstream of the endostatin gene, and the human plasminogen signal sequence was included upstream of the angiostatin gene (Fig. 1*A*). Because previous studies using purified recombinant angiostatin and endostatin indicated that a combination of both the factors resulted in significant therapeutic benefit, we also produced a vector capable of bicistronic expression of angiostatin and endostatin using an IRES sequence (Fig. 1*A*), to determine whether long-term expression of these two antiangiogenic factors from the same vector would provide greater advantage over vectors encoding a single factor. The rAAV were packaged in a helpervirus-free system and purified using discontinuous gradient centrifugation and affinity chromatography. The titer of the vectors ranged between 1–5 $\times$ 10^{12} particles/ml.

Expression of Angiostatin and Endostatin as Secretory Proteins. The purified rAAV were tested in 293 cells for expression of antiangiogenic factors as secretory proteins in the medium. Cells were either mock-transduced or transduced with rAAV encoding endostatin alone, angiostatin alone, or the bicistronic vector containing both angiostatin and endostatin. After transduction, the cells were cultured for 48 h. The conditioned medium was harvested, concentrated, and subjected to SDS-PAGE and Western blot analysis using monoclonal antibody specific for either human angiostatin or endostatin. Results, shown in Fig. 1, *B* and *C*, documented expression of both angiostatin and endostatin as secretory proteins after rAAV-mediated gene transfer *in vitro*. The endostatin and angiostatin antibodies recognizing 20 and 38 kDa proteins, respectively, also indicated the specificity of detection. Of interest, the detected amount of both factors was comparable when transduced as a single transgene or a bicistronic cassette. As expected, no signal was seen in conditioned medium from mock-transduced 293 cells.

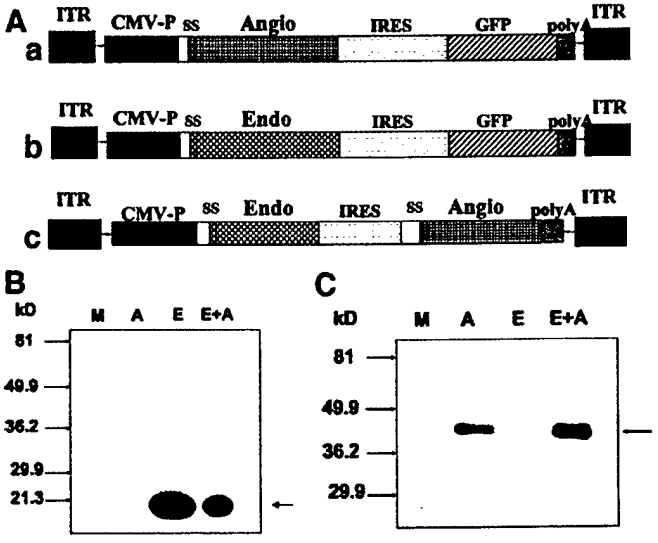

Fig. 1. Recombinant adeno-associated virus 2 (rAAV) encoding endostatin and/or angiostatin and Western blot analysis for transgene expression. *A*, rAAV containing human angiostatin (*Angio*) or endostatin (*Endo*) gene either in different vectors (*a* & *b*), or as bicistronically expressed proteins from a single vector (*c*) were subcloned under the control of the cytomegalovirus promoter (CMV-P). Secretory signal sequences (SS) of either interleukin 2 or human plasminogen gene were included upstream of the angiostatin and endostatin genes respectively for systemic secretion. ITR represents the inverted terminal repeat sequences of AAV. *B* and *C*, 293 cells were either mock-transduced (*M*) or transduced with rAAV encoding angiostatin (*A*), endostatin (*E*) or a bicistronic cassette encoding both endostatin and angiostatin (*E*+*A*). Forty-eight h after transduction, the culture supernatant was analyzed by Western blots using antibodies specific for human endostatin (*B*) or angiostatin (*C*). *polyA*, polyadenylation signal sequence.

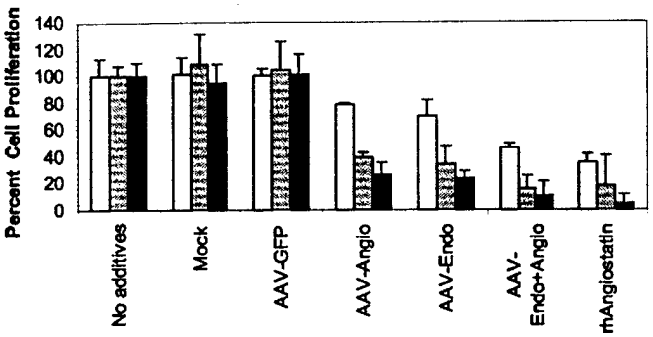

Fig. 2. Endothelial cell proliferation assay to determine the biological activity of endostatin (Endo) and angiostatin (Angio) produced after recombinant adeno-associated virus 2 (rAAV) transduction. rAAV encoding green fluorescent protein (GFP), Endo, Angio, or Endo + Angio was transduced into 293 cells at a multiplicity of 100. Forty-eight h later, 1 (□), 10 (▨), or 25 (■) μl of supernatant from the infected cells was tested on human umbilical vein endothelial cell (HUVEC) cultures. Cell proliferation was determined 72 h later using 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide colorimetric assay and results expressed as percentage of control HUVEC with no treatment.

Characterization of Biological Activity of rAAV-Produced Angiostatin and Endostatin. We next determined the biological activity of rAAV-expressed angiostatin and endostatin. Because our strategy for AAV-mediated gene therapy was to express the antiangiogenic factors as secreted proteins, the *in vitro* evaluation of biological activity was performed similar to *in vivo* strategy. rAAV encoding antiangiogenic factors were transduced into 293 cells, and the transgene products were obtained as secreted protein in the supernatant. Different concentrations of the supernatants were then added to early passage HUVEC cultures grown in the presence of 10 ng/ml VEGF. Differences in cell proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide colorimetric assay. Results (Fig. 2) demonstrated a significant inhibition of HUVEC proliferation by conditioned media from both AAV-endostatin and AAV-angiostatin-transduced 293 cells as compared with medium from mock-transduced cells ($P < 0.002$). The results also showed a dose-dependent growth-inhibitory effect with increasing amounts of conditioned medium. Interestingly, there was no statistically significant augmentation in the inhibitory effects on HUVEC proliferation in conditioned medium from cells transduced with angiostatin-endostatin bicistronic construct as compared with constructs expressing only a single factor ($P > 0.05$). There was no inhibitory effect on HUVEC proliferation when conditioned medium obtained from rAAV-GFP-transduced cells was used, demonstrating specificity of the rAAV-expressed antiangiogenic proteins. Purified recombinant human angiostatin was used as a positive control. Although we did not quantitatively determine the amount of angiostatin or endostatin in the conditioned media in these experiments, results indicated inhibitory effects comparable with that of 25 ng/ml purified angiostatin protein when 10–25 μl of conditioned medium was tested (Fig. 2).

***In Vivo* Vector Administration and Development of Xenograft Tumor Model.** As a model to evaluate the *in vivo* efficacy of AAV-mediated long-term expression of angiostatin and endostatin, we used athymic (nude) mouse s.c. xenografts of an angiogenesis-dependent human ovarian cancer cell line, SKOV3.ip1. Pilot studies indicated palpable SKOV3.ip1 tumors in nude mice 8–10 days after s.c. injection of 10^7 SKOV3.ip1 cells (data not shown). Because the present studies were primarily designed to evaluate the potential of AAV-

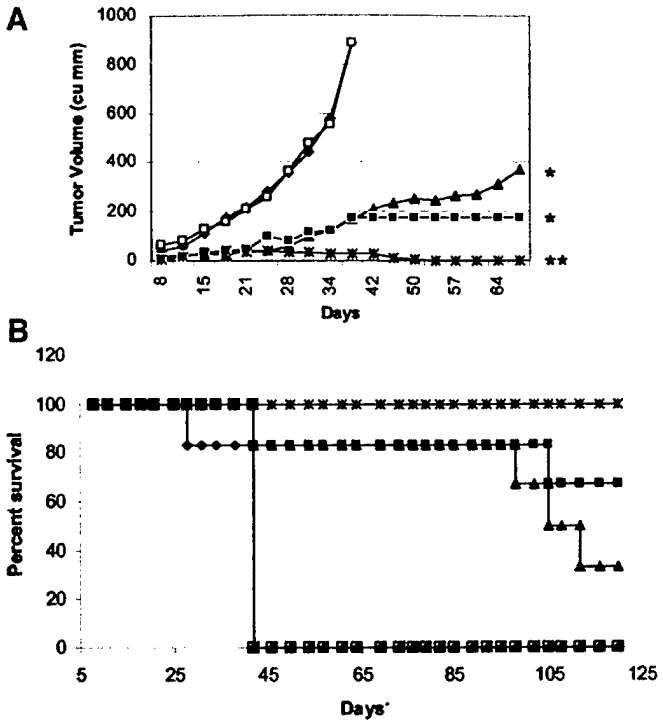

Fig. 3. Growth of SKOV3.ip1 tumor xenografts in athymic nude mice after injection of recombinant adeno-associated virus 2 (rAAV) encoding endostatin and angiostatin. Mice received i.m. injection with saline (♦), or with 3×10^{11} particles of AAV-GFP (□), AAV-endostatin (▲), AAV-angiostatin (■) or AAV-endostatin + angiostatin (*), and 3 weeks later challenged by bilateral s.c. injection of 10^7 SKOV3.ip1 cells (six mice/group). *A*, average tumor volume in each group. The * and ** denote $P < 0.001$ and $P < 0.0001$, respectively, by comparison to naïve and AAV-GFP control groups. *B*, tumor-free survival. *GFP*, green fluorescent protein

mediated antiangiogenic gene therapy as a preventive therapy against tumor recurrence, animals first received injection with rAAV encoding angiostatin or endostatin. Because optimal expression of rAAV transgenes after i.m. injection is reached at about 3 weeks after administration, tumor challenge was done 3 weeks after vector. Each animal received two tumor implants on bilateral flanks. All naïve mice and control mice receiving injection with rAAV-GFP developed palpable tumors by day 8 after tumor cell implantation. Animals that developed tumors were monitored until tumor volume reached 1800 mm^3, and then animals were euthanized. Tumor-free animals were monitored up to 130 days before terminating the experiment.

Inhibition of Tumor Growth after Injection of rAAV Expressing Endostatin, Angiostatin, or Endostatin Plus Angiostatin. The results of the *in vivo* studies are shown in Fig. 3. Growth kinetics of SKOV3.ip1 tumors are shown in Fig. 3*A*, and tumor-free survival of mice is presented in Fig. 3*B*. There was a significant protective effect of AAV-mediated antiangiogenic gene expression with both endostatin and angiostatin compared with control or AAV-GFP-treated animals, as assessed by either tumor-free survival or tumor-growth kinetics. However, when both the factors were expressed from the bicistronic vector, the effect was more pronounced than therapy with vector expressing only a single factor ($P < 0.0001$). Protection by AAV-endostatin alone was significantly less than by AAV-angiostatin alone (Fig. 3*B*). Interestingly, serum levels of endostatin or angiostatin were comparable in all mice within the same group irrespective of observed tumor growth. Mean tumor volumes were significantly less in animals that received angiostatin or endostatin gene therapy compared with either naïve animals or those given rAAV-GFP ($P < 0.001$). Of note, in mice that received vector encoding both angiostatin and endostatin, only one animal developed a palpable tumor, which regressed after day 30 (data not shown), after which all mice in that treatment group remained tumor free.

Serum ELISA for Circulating Angiostatin and Endostatin Levels. To determine the circulating levels of serum angiostatin, we developed a sandwich ELISA as described in the "Materials and Methods." Using purified recombinant human angiostatin, the sensitivity of the ELISA was demonstrated to be 12 ng/ml. Serum samples were obtained from all animals (*a*) before tumor cell implantation and (*b*) before sacrifice of the animals because of either tumor burden or termination of the experiment. Results indicated that serum angiostatin levels remained stable up to 130 days after AAV-angiostatin injection, and levels were comparable with those in mice that received injection with the bicistronic AAV construct expressing both angiostatin and endostatin (Fig. 4*A*). Serum endostatin levels were determined using a commercial kit and demonstrated a pattern of endostatin expression that was similar to that for angiostatin (Fig. 4*B*). The serum angiostatin and endostatin levels appeared to have stabilized before the tumor cell implantation on day 21 after vector administration.

Long-Term Retention and Expression of rAAV-Endostatin/Angiostatin. An advantage of rAAV vectors in muscle-based gene therapy is the sustained presence of transgene(s) as either integrated or episomal copies for long-term expression. Because antiangiogenic gene therapy is directed toward inhibiting proliferation of tumor neovasculature and not tumor cells directly, a critical requirement for antiangiogenic therapy is stable expression of these factors at therapeutic levels. Studies have reported that administration of rAAV into muscle not only results in long-term expression of the transgene but also that vector administered this way does not elicit host immune response against the transgenic protein, which would otherwise eliminate transgene expressing cells (31, 32). Thus, in the present study,

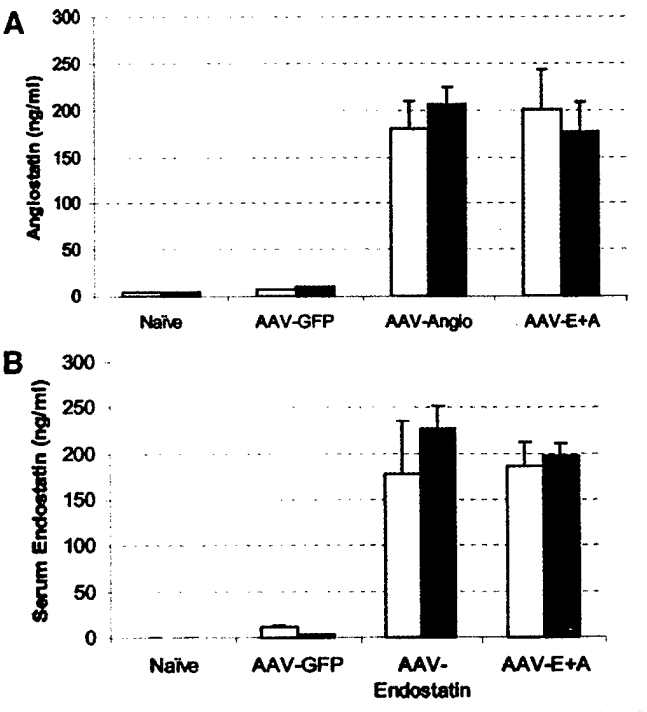

Fig. 4. Levels of human endostatin and angiostatin in mouse serum after injection of recombinant adeno-associated virus 2 (rAAV) encoding green fluorescent protein (GFP), endostatin (E), or angiostatin (Angio or A). Serum samples were obtained before tumor challenge (□) and at the time of sacrifice of mice because of either tumor burden or termination of the experiment (■). *A*, a sandwich ELISA was developed for measuring human angiostatin. *B*, ELISA for human endostatin used a commercial kit. Data show mean ± SD of calculated concentrations of each factor (mean of triplicates).

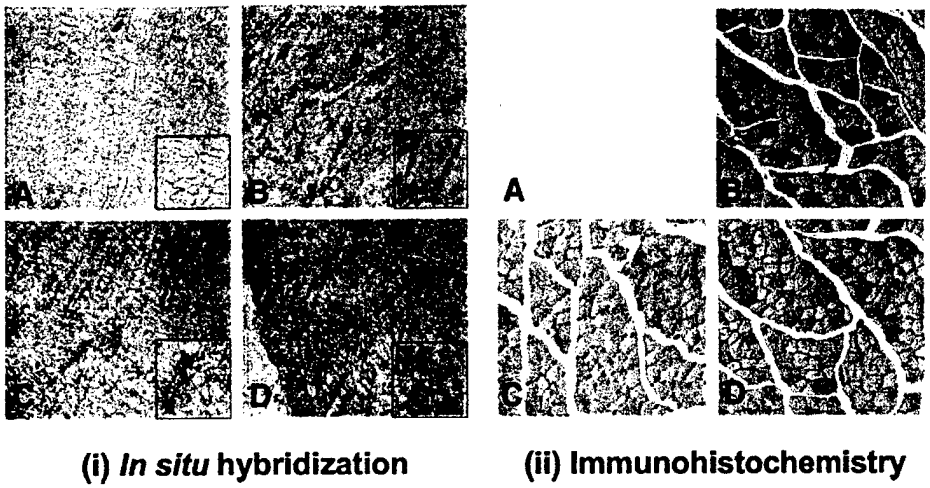

Fig. 5. *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Five-μm sections of injected muscles were used for *in situ* hybridization (*left panels*) and immunohistochemistry (*right panels*). *A*, uninjected. *B*, AAV-endostatin. *C*, AAV-angiostatin. *D*, AAV-endostatin + angiostatin bicistronic. For *in situ* hybridization, *inserts* show a larger magnification of representative areas. For immunohistochemistry, *A*, *B*, and *D* were stained with antibody specific for human endostatin, and *C* was stained with antibody specific for human angiostatin.

we used skeletal muscle as a target tissue for stable expression of rAAV and systemic secretion of endostatin and angiostatin using a signal peptide. To demonstrate persistence and expression of AAV transgene, we performed *in situ* hybridization and immunohistochemistry, respectively. Because the kringle 1–4 region of plasminogen (representing angiostatin) and the internal collagen XVIII domain (representing endostatin) show significant homology between human and mouse, we used a DIG-labeled probe to the transgene CMV promoter region for *in situ* hybridization analysis. Also, because all of the vector constructs used in the study contained the CMV promoter, it was possible to use the same probe for detection of vector genome in all treatment groups. Results (Fig. 5, *left*) demonstrate the persistence of AAV transgene in skeletal muscle of mice after 130 days. Although the *in situ* data cannot demonstrate stable integration of the vector into cell genomes, based on previous preclinical and clinical studies, it is clear that rAAV administered in skeletal muscle establishes long-term presence (33).

Immunohistochemistry was performed in paraffin sections of the transduced muscle to determine the expression of endostatin/angiostatin. Representative results (Fig. 5, *right*) indicate the presence of each antiangiogenic factor in the AAV-injected muscle tissue. On the basis of the immunohistochemistry results, it is most likely that the cells showing positive staining for angiostatin or endostatin are only those in which *in situ* hybridization identified the vector genome.

DISCUSSION

Antiangiogenic therapy is a promising approach for the control of solid tumor growth and metastasis. Although several drugs have shown promise in controlling tumor neovasculature, a major problem in pharmacotherapy is side effects of constant drug administration and the limited half-life of antiangiogenic proteins (34). Gene therapy, on the other hand, offers advantages of maximizing cost effectiveness and maintaining sustained levels of antiangiogenic factors, which may enhance antitumor efficacy.

Although many factors are known to play important roles in new blood vessel formation, two major factors that play a key role are VEGF and fibroblast growth factor. Previous reports have presented contradictory results on the efficacy of antiangiogenic factors in controlling tumor growth in preclinical studies and clinical trials (19, 22, 35, 36). In the present study, we evaluated the potential of human angiostatin and endostatin using rAAV for stable transfer of genes encoding these factors. The major advantages of AAV vectors are nonpathogenicity, less immunogenicity, and long-term stable expression of the antiangiogenic factors.

Because the initial discovery that biologically driven antiangiogenic agents are much safer and effective, studies have focused on testing their potential in preclinical and clinical applications (7–23). However, the possible mechanisms of action of these factors have only recently begun to be uncovered. Whereas angiostatin, a proteolytic fragment of plasminogen, acts by binding to $\alpha v\beta 3$ integrin (37, 38), endostatin, an internal fragment of collagen XVIII, is believed to act by binding to tropomyosin, integrins, and matrix metalloproteases (39–41). Thus, it is clear that these two factors act on distinct pathways and targets. Hence, a treatment using these two factors should have an additive or even synergistic effect compared with therapy using only one factor. Results of our *in vivo* studies clearly demonstrate this. An apparently synergistic tumor protective effect was observed in mice that received the bicistronic vector encoding both angiostatin and endostatin as compared with mice that received vectors encoding only one of the factors.

The effect of a combination treatment of endostatin and angiostatin over that using a single factor therapy was reported previously using purified protein therapy (14). Interestingly, results of previous studies have suggested differences in the efficacy of protein as compared with gene therapy approaches. Whereas administration of 20 mg/kg endostatin as denatured purified protein was effective in controlling the growth of an angiogenesis-dependent Lewis lung carcinoma (11), gene therapy approaches using adenoviral or retroviral vectors produced only a modest therapeutic effect (42, 43). The reasons for this could be different pharmacokinetics and tissue distribution of the denatured purified protein compared with the *in vivo*-expressed factor or the nature of vectors used.

Despite encouraging results from preclinical studies using protein therapy, a major concern for clinical applications is the limited half-life of the purified proteins. Furthermore, the half-lives of endostatin and angiostatin are different, which may complicate drug scheduling. Stable gene therapy approaches such as described here can overcome these limitations by maintaining stable systemic levels of both angiostatin and endostatin. Although rAAV vectors have been used in many preclinical and a few human clinical studies, a majority of these applications have been in the context of genetic metabolic defects to provide long-term expression of defective enzymes/factors (33, 44). However, a few studies with antiangiogenic factors have provided promising preclinical data, indicating the potential of long-term gene therapy targeting the inhibition of tumor neovasculature (45, 46). Our

data provide evidence that synergy between angiostatin and endostatin delivered by AAV-dependent gene transfer will be clinically relevant to control recurrence and metastasis of primary cancers.

Almost all early preclinical studies of antiangiogenic tumor gene therapy have been performed in immunodeficient mice and, hence, cannot predict the role of host immunity on long-term transgene expression. A potential advantage of rAAV is the proven long-term *in vivo* expression of AAV-encoded transgenes administered in skeletal muscle of immunocompetent individuals. Because rAAV does not encode any viral proteins, host immune response against the vector is minimal (31, 32). In addition, potent antigen presenting cells, especially dendritic cells, are not transduced efficiently by rAAV. Undiminished expression of AAV-transgene as a secreted protein in muscle has been recorded for over 4 years in immunocompetent animals (47).

Persistence and stable expression of AAV-encoded antiangiogenic factors is evident from our results of ELISA for serum levels and *in situ* DNA hybridization studies of injected muscle tissue. Recent studies using intratumoral administration of rAAV encoding endostatin in a mouse glioma model and i.m. administration of rAAV-endostatin in a colorectal cancer model have also shown therapeutic efficacy (45, 46). A limitation in the intratumoral administration of rAAV is the poor transduction efficiency in primary tumors as well as the lack of accessible tumor sites for treatment in many patients. Thus, administration of the vector in skeletal muscle may represent a preferred approach, especially for treatments in the setting of minimal residual disease.

On the other hand, if constant systemic levels of antiangiogenic factors become toxic to the experimental animals or patients, the approach presented in this study may not prove superior over localized production of the factors within tumors. In such situations, development of targeted AAV with increased infectivity to tumor cells may be highly beneficial. Accumulation of antiangiogenic factors in other organs because of unregulated expression may also lead to ischemic conditions or impair wound healing. Thus, future studies will be necessary to test the safety of long-term expression of angiostatin and endostatin, and the development of vectors allowing regulated expression of transgenes by inducible promoters, for example, may be warranted for full development of this genetic therapy (48, 49).

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Anti-angiogenic cancer gene therapy by adeno-associated virus 2-mediated stable expression of the soluble FMS-like tyrosine kinase-1 receptor

(*Short title*: AAV2-mediated sFlt-1 cancer gene therapy)

Gandham Mahendra[1], Sanjay Kumar[1], Tatyana Isayeva[1], Parameshwar Mahasreshti[2,4], David T. Curiel[1,3,4], Cecil R. Stockardt[1], William E. Grizzle[1], Vidya Alapati[1], Raj Singh[1], Gene P. Siegal[1,4], Sreelatha Meleth[3], and Selvarangan Ponnazhagan[1,4*]

Departments of Pathology[1], Obstetrics and Gynecology[2], Medicine[3] and Gene Therapy Center[4]

The University of Alabama at Birmingham, Birmingham, AL 35294

***Corresponding author**

Selvarangan Ponnazhagan, Ph.D.

Department of Pathology, LHRB 513

701, 19th Street South

University of Alabama at Birmingham

Birmingham, AL 35294-0007

Phone: (205) 934-6731

Fax: (205) 975-9927

E-mail: sponnazh@path.uab.edu

Abstract

Anti-angiogenic gene transfer has the potential to be more efficacious than protein-based therapies or pharmacotherapies for the control of solid tumor growth, invasion and metastasis. For a sustained anti-angiogenic effect, a vector capable of long-term expression without vector-associated immunity or toxicity is advantageous. The present study evaluated the potential of a recombinant adeno-associated virus-2 (rAAV) encoding the human soluble FMS-like tyrosine kinase receptor 1 (sFlt-1), which functions by both sequestering vascular endothelial growth factor (VEGF) and forming inactive heterodimers with other membrane-spanning VEGF receptors, *in vitro* and *in vivo*. Results indicated significant growth inhibitory activity of the transgenic factor in a human umbilical vein endothelial cell proliferation assay *in vitro* and protection against the growth of an angiogenesis-dependent human ovarian cancer cell line, SKOV3.ip1, xenograft *in vivo* with increased disease-free survival. Stable expression of the secretory factor and transgene persistence were confirmed by immunohistochemistry and *in situ* hybridization analyses respectively. Increased therapeutic effects on both the growth index of the implanted tumor cells and tumor-free survival also correlated with an increasing dose of the vector used. These studies indicate that rAAV-mediated sFlt-1 gene therapy may be a feasible approach for inhibiting tumor angiogenesis, particularly as an adjuvant/preventive therapy.

Introduction

A better understanding of the interaction of normal host and tumor cells has provided clues towards developing novel therapies for cancer. Cancer gene therapy targeting non-neoplastic cells has recently shown greater potential against tumor growth and metastasis in preclinical models. One of the promising areas of cancer therapy is targeting the growth of tumor-associated endothelium, which provides anchorage and nourishment for the growth of solid tumors through the process of angiogenesis (1). It has been clearly established that in the absence of angiogenesis, tumors fail to grow beyond 2-3 mm^3 in size and remain dormant (1,2). Thus, by providing anti-angiogenic signals through sustained systemic expression of the inhibitory factors at therapeutic levels, it may be possible to control the growth of solid tumors. Further, anti-angiogenic gene therapy can be combined with conventional therapies such as radiation or chemotherapy or newer therapies including immunotherapy for synergistic effects (3).

For sustained expression of anti-angiogenic factors at a therapeutic level by gene transfer, an important requirement is the use of vectors capable of stable expression without the possibility of elimination of transgene-positive cells by T cell-mediated cytolysis. Adeno-associated virus (AAV)-based vectors have shown the potential for long-term expression of therapeutic genes without such vector-associated immunity or toxicity (4-7). Since anti-angiogenic gene therapy mandates a sustained expression of the anti-angiogenic factors at a therapeutic level, rAAV is an ideal vector to accomplish this.

Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) is a splice variant of the vascular endothelial growth factor (VEGF) receptor Flt-1, lacking the transmembrane and cytoplasmic domains (8). VEGF is a potent angiogenic factor, and overexpression of VEGF has been reported to be associated with poor prognosis in many human cancers (9-13). The VEGF antagonistic activity of sFlt-1 is effected both by forming inactive heterodimers with membrane-spanning VEGF receptor and by sequestering VEGF in a dominant negative manner thereby inhibiting the downstream VEGF signaling cascade following receptor-mediated internalization (8). Thus, use of sFlt-1 for downregulating VEGF signaling at two different steps would maximize the process of inhibiting tumor neovascularization and associated tumor growth. The present study demonstrates that stable expression of sFlt-1 following rAAV-mediated gene transfer provides significant protection against the growth of an angiogenesis-dependent human ovarian cancer cell line in a mouse xenograft model suggesting its potential application in anti-angiogenic cancer gene therapy in humans.

Materials and Methods

Cells and reagents. The human embryonic kidney cell line, 293, was purchased from the ATCC and maintained in Iscove's Modified Essential Medium supplemented with 10% fetal bovine serum. Human ovarian cancer cell line SKOV3.ip1 was a kind gift of Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX) and was maintained as before (14). Primary human umbilical vein endothelial cells (HUVEC) were obtained from Dr. Francoise Booyse (The University of Alabama at Birmingham, AL) and maintained as before (15). Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corporation (Madison, WI). A mouse monoclonal anti-human VEGF receptor-1 (Flt-1 receptor) antibody, which recognizes the extracellular domain of Flt-1 (represented in the sFlt-1) was purchased from Sigma Chemicals (V4262, St. Louis, MO). A rat anti-mouse monoclonal antibody for CD31 (PECAM-1) was purchased from Chemicon International (CBL1337, Temecula, CA). Secondary antibodies and color reagents were purchased from Abcam Inc., Cambridge, MA and Amersham, Piscataway, NJ. Purified recombinant human sFlt-1 was purchased from R&D Systems Inc., Minneapolis, MN.

Construction of recombinant plasmids, production and purification of rAAV. All rAAV plasmids were constructed using pSub201 as the backbone (16). cDNA encoding human sFlt-1 was cloned from a human placental cDNA library as described (17). The coding sequences were initially subcloned in a

mammalian expression vector pCI (Promega, Madison, WI) under the control of the CMV promoter and a cassette comprising the CMV promoter, sFlt-1 gene and polyadenylation signal sequence was isolated and subcloned in pSub201, replacing the AAV-2 genes. Packaging of rAAV-sFlt-1 was accomplished in an adenovirus-free system as described (18). Purification of the virions was carried out in a discontinuous iodixanol gradient centrifugation followed by affinity purification in heparin affinity column. The particle titer of the purified virions was determined by quantitative slot blot analysis as described (19,20).

Western blot analysis. Western blot analysis was performed using conditioned media obtained from rAAV-transduced 293 cells. Briefly, conditioned media collected following mock-transduction (without any vector) or rAAV-sFlt-1 transduction was concentrated 5-fold and 20 µl from each aliquot was electrophoretically separated in a 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). The gel was transferred to a PVDF membrane and immunodetection of the proteins was performed using a monoclonal antibody directed against the extracellular region of the human sFlt-1. A goat anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP), was used as a secondary antibody. Detection of the bands was performed using an enhanced chemiluminescent (ECL) system as described (21).

Endothelial cell proliferation assay. Early passage HUVEC were seeded in gelatin-coated 96-well tissue culture plates at a density of $5x10^3$ cells per well

and grown in EGM-2 medium containing hydrocortisone, human FGF-β, VEGF, ascorbic acid, heparin, human EGF and 10% fetal bovine serum (Clonetics Corp., San Diego, CA). Twenty-four hr later, the medium was changed and 100 µl of fresh medium containing the same additive plus 20 µl of conditioned medium obtained from 293 cells that were either mock-transduced or transduced with 100 MOI of rAAV-sFlt-1. As a positive control, purified recombinant human angiostatin (R&D Systems, Minneapolis, MN) was added in a separate well at a concentration of 20 µg/ml. Seventy-two hours later, the cells were fixed in 10% buffered-formalin and stained with 1% crystal violet in 70% ethanol as described (22) to analyze the rate of proliferation.

***In vivo* studies.** Six-week old female athymic nude mice were purchased from the Fredericks Cancer Institute, NCI, and housed in the animal facility of the University of Alabama at Birmingham (UAB). The animals were maintained following the guidelines of the Institutional Animal Care and Use Committee (IACUC) after all experimental procedures were approved by both the UAB-IACUC and the Occupational Health and Safety Department. The animals were divided into six groups consisting of six mice per group. $3x10^{11}$ particles of rAAV encoding either GFP, or sFlt-1, suspended in normal saline, were injected in a volume of 50-100 µl in the quadriceps muscle of the hind limb. Naïve animals did not receive any vector. Three weeks after vector administration, each mouse was implanted with 10^7 SKOV3.ip1 cells, subcutaneously. A total of two injections were delivered per mouse, one in each flank. Tumor size was

measured twice a week with a digital caliper for two-dimensional longest axis (L in mm) and shortest axis (W in mm), and tumor volume calculated using the formula: volume in $mm^3 = (L \times W^2)/2$. If the tumor growth exceeded 1800 mm^3, the animals were euthanized. The surviving mice were sacrificed by the end of 130 days after tumor cell implantation, and the experiment terminated. At the time of sacrifice either due to tumor burden or termination of the experiment, both the liver and regions of the quadriceps muscle at the site of sham or vector injection were isolated and processed for total DNA isolation, histology, immunohistochemistry and *in situ* hybridization.

Histological analyses of liver tissue. Liver tissues were harvested from naïve or rAAV-sFlt1 treated mice. The tissues were immediately fixed in 10% buffered-formalin (pH 7.0) and embedded in paraffin following standard processing methods. Sections of 5 μm thickness were cut, deparaffinized in xylene and dehydrated in alcohol. The slides were H&E stained and mounted with cover slips. Analysis of the stained sections was carried out by a senior histopathologist by standard bright-field microscopy (23).

PCR analysis for vector genome. Total DNA was isolated from naïve or rAAV-sFlt1 administered mouse muscle using TRIizol reagent (GIBCO-BRL). PCR amplification was carried out in a 30 cycle reaction using a primer-pair, specific for the amplification of the vector genome. The forward primer consisted sequence of CMV promoter and the reverse primer that of the human sFlt-1. The

primer sequences were: forward primer 5'-TAAGCAGAGCTCGTTTAGTGAACCGT-3' and reverse primer, 5'-TACTCACCATTTCAGGCAAAGACCAT-3'. The amplified product (548 bp) was electrophoretically separated on 1% agarose gels and the bands visualized by ethidium bromide staining.

Immunohistochemistry. The quadriceps muscle of mice were harvested under anesthesia and fixed immediately in alcoholic-formalin (PenFix; Richard-Allan, Kalamazoo, MI) for 18 hrs at room temperature. The tissues were dehydrated in graded alcohol and embedded in paraffin. Five micrometer sections, prepared on glass slides were deparaffinized in xylene and rehydrated via ethanol and placed in PBS. Antigen retrieval was performed by boiling the sections for 10 min in 0.01 M citrate buffer (pH 6.0) in a microwave oven. All sections were pretreated with a 3% aqueous solution of H_2O_2 for 5 min to quench endogenous peroxidase. Sections were then treated with 3% goat serum for 1 hr at room temperature to reduce non-specific staining followed by 1 hr incubation with a human monoclonal anti-VEGF receptor-1 antibody, which recognizes the extracellular domain of the human VEGF receptor-1 (present in the sFlt-1). The antibody was used at a concentration of 10 µg/ml. The remainder of staining procedure was performed using a Universal Mouse Kit (Biogenex, Sam Ramon, CA), which contained biotinylated goat linking antibody to mouse immunoglobulins and a HRP-streptavidin complex. Diaminobenzidine tetrahydrochloride (DAB) was used as a substrate for the visualization of antigen-

antibody complex. Slides were minimally counterstained with hematoxylin. To determine the degree of neovasculature, tumor tissues from naïve and rAAV-sFlt-1 treated mice, harvested at the same time, were processed as above. Detection of blood vessel was performed with a rat anti-mouse CD31 monoclonal antibody at a concentration of 10 µg/ml. The number and area of blood vessels in each group were counted in at least ten different areas in a double-blinded method.

***In situ* hybridization.** A digoxigenin (DIG)-labeled DNA probe containing the CMV promoter sequence was generated by PCR using the PCR-DIG labeling mix^{Plus} (Roche Molecular Diagnostics) following the manufacturer's protocol. Formalin-fixed tissues were sectioned to five micron thickness, deparaffinized in xylene and rehydrated through a series of graded-ethanol and PBS. Slides were then treated with 0.01M citrate buffer, pH 6.0 at 42°C for 3 hrs. Prehybridization was performed at 65°C for 2 hrs in hybridization solution (ULTRAhyb^{TM}, Ambion, TX). The hybridization reaction was carried out with approximately 400 ng/ml of the DIG-labeled DNA probe at 65°C overnight. After thoroughly washing the excess probe, immunohistochemical detection of hybridization signals was performed using the DIG nucleic acid detection kit (Roche Molecular Diagnostics, Indianapolis, IN). Counterstaining of the slides was carried out with a diluted eosin solution for 1-2 minutes and then mounted with Crystal/Mount (Biomeda, Forest City, CA).

Statistical analyses. The association of the presence or absence of tumor with differing treatment conditions was tested for statistical significance by using the *Chi*-square test. The distribution of the tumor volume was measured against a normal (Poisson's distribution) following log transformation. In a general linear mixed model of the data, the dependent variable was the logged tumor volume and the predictors were treatment, day after treatment and "an interaction event" between treatment and day 0. All three were subsequently shown to be statistically significant predictors. The values of blood vessel area from naïve and rAAV-sFlt-1 were compared using Student's *t* test. *P* values <0.05 were considered to indicate significant difference between data sets.

Results

Generation of high-titer rAAV encoding human sFlt-1 for skeletal muscle injection. Previous studies have established sustained expression of rAAV transgenes in skeletal muscle without diminution of expression or deterioration of transgene-expressing cells by cytolytic T cells. Hence, in the present study, we have chosen skeletal muscle as a platform for rAAV administration and production of sFlt-1 as a secretory protein. The human sFlt-1 cDNA was isolated from a HUVEC cDNA library (17) and subcloned in an rAAV vector under the control of the human cytomegalovirus immediate early promoter (CMV) (Figure 1). The rAAV was packaged in a helper virus-free system and purified using discontinuous iodixanol gradient centrifugation and affinity chromatography. The titer of the vector ranged between 1-5x10^{12} particles/ml.

Determination of sFlt-1 expression as a secretory protein. The rAAV-sFlt-1 was tested in 293 cells for the extracellular secretion of the factor. Since the cDNA of cloned sFlt-1 contained the native VEGF receptor secretory signal, no additional modifications were performed to achieve extracellular transport of sFlt-1 from the transduced cells. The cells were either mock-transduced or transduced with 100 multiplicity of infection (MOI) of the rAAV encoding sFlt-1. The conditioned medium, obtained 48 hrs after transduction, was concentrated five-fold and separated on SDS-PAGE and Western blot analysis performed using a monoclonal antibody, which recognizes human sFlt-1. Results, shown in Figure 2, clearly indicated a high-level expression of human sFlt-1 as a secretory

protein following rAAV transduction in 293 cells. The antibody, recognizing sFlt-1 in supernatants obtained only from the rAAV-transduced 293 cells but not from the mock-transduced cells also indicated the specificity of detection.

Determination of biological activity of sFlt-1 following rAAV transduction. In the next set of experiments, we determined the biological activity of rAAV-expressed sFlt-1. We based our therapeutic approach on the well established property of rAAV transgene expression as a secretory protein in skeletal muscle. Thus, the *in vitro* evaluation of the biological activity was performed mimicking the *in vivo* strategy. The rAAV containing sFlt-1 gene was transduced into 293 cells and the transgene product was obtained as a secreted protein in the supernatant. The supernatant was added to early passage HUVEC, grown in the presence of 10 ng/ml VEGF. Seventy two hours later, the cells were fixed and stained with crystal violet. The proliferation index was determined by microphotography. Results, given in Figure 3, indicated a significant inhibition of HUVEC proliferation following the addition of supernatant from rAAV-sFlt1 transduced cells but not from the mock-transduced cells. As a positive control, purified recombinant human angiostatin was used at a concentration of 20 μg/ml, which also showed a significant inhibition of HUVEC proliferation. Highest inhibition, comparable to that from purified angiostatin, was observed with 25 μl supernatant indicating that an amount of approximately 125 ng/ml is produced in the supernatant of rAAV-sFlt-1 transduced cells.

Development of a xenograft tumor model and vector treatment. As a model system to evaluate the *in vivo* anti-angiogenic effects of rAAV-mediated long-term expression of sFlt-1, we developed subcutaneous tumors with an angiogenesis-dependent human ovarian cancer cell line SKOV3.ip1. Our pilot studies indicated that palpable tumors begin to appear 8-10 days following subcutaneous implantation of 10^7 SKOV3.ip1 cells in nude mice (data not shown). Since we based the present studies to evaluate the potential of AAV-mediated sFlt-1 gene therapy as a possible preventive therapy against the growth and recurrence of solid tumors, the animals were first injected with rAAV encoding sFlt-1. Also because optimal expression of rAAV transgenes is not achieved until at least two weeks after vector administration, tumor challenge was performed 3-weeks after vector administration. Each animal received an injection in both flanks. All the naïve animals and rAAV-GFP administered animals developed palpable tumors by 8 days after injection. The animals that developed tumors were monitored until the tumor volume reached 1800 mm^3 and euthanized according to the IACUC approved guidelines. Tumor-free animals were monitored for 130 days before terminating the experiment.

Evidence of transgene persistence and expression following rAAV-sFlt-1 therapy *in vivo*. A major advantage of using rAAV in muscle-based gene therapy is the stable retention of transgene(s) as either integrated or episomal copies for long-term expression. Since anti-angiogenic gene therapy is directed towards inhibiting proliferation of tumor neovasculature and not tumor cells

directly, an important requirement for anti-angiogenic therapy is stable expression of these factors at therapeutic levels. Studies have reported that administration of rAAV in muscle not only results in long-term expression of the transgene but also that vector administered in this way does not elicit any host immune response against the transgenic protein, which would otherwise eliminate transgene-expressing cells (4,24). Thus, in the present study, we sought to achieve this result by using skeletal muscle as a target tissue for stable integration of rAAV and systemic secretion of sFlt-1. To demonstrate the long-term persistence of the AAV transgene, we performed genomic PCR and *in situ* hybridization, and to determine the systemic secretion of sFlt-1 from transduced muscle, we performed immunohistochemistry.

PCR analysis of vector genome was performed using total DNA isolated from naïve or rAAV-sFlt-1 treated mouse muscle. The forward and reverse primers consisted sequences of CMV promoter and human sFlt-1 coding sequences respectively. An amplification product of 548 base-pairs, only in rAAV-sFlt-1 treated mice [Figure 4(i)] confirmed the persistence of vector genome. For *in situ* hybridization analysis, we used a digoxigenin (DIG)-labeled CMV promoter sequence as the probe. Results, shown in Figure 4(iii), also demonstrate the persistence of the AAV transgene in the skeletal muscle of only the vector-treated mice after 130 days. The use of CMV-promoter sequence as a probe in the *in situ* hybridization experiment also eliminated the possibility of hybridization signal from transgenic mRNA transcripts as well as cellular Flt-1 genomic DNA. Although the results of PCR or *in* situ hybridization do not confirm

the integration of the rAAV genome into the host, increasing evidence indicate that only a small amount of rAAV transgenes integrate *in vivo* (25). Nonetheless, in skeletal muscle, concatemerization of the rAAV genome allows long-term retention of transgenes as circular episomes (6,7).

Immunohistochemistry was performed in paraffin sections of the transduced muscle and tumor tissues from naïve and rAAV-sFlt-1 treated mice to determine the expression of human sFlt-1 and degree of neovasculature respectively. Data, shown in Figure 4B, indicates systemic secretion of human sFlt-1 from the quadriceps muscle, only from rAAV-sFlt1-treated mice. Since the present study was performed as a preventive approach, tumor growth was not observed in disease-free animals in the sFlt-1 group. However, when the tumor sections were stained with CD31 antibody, there was a significant decrease ($P<0.04$) in the blood vessel area in rAAV-sFlt-1 group suggesting the inhibitory effect on blood vessel growth.

Stable expression of sFlt-1 inhibits tumor growth *in vivo*. The results of the *in vivo* studies on growth kinetics of SKOV3.ip1 cells and tumor-free survival with a vector dose of $3x10^{11}$ particles are given in Figures 5 and 6 respectively. However, when a vector dose of $3x10^{10}$ particles was used, although there was a modest increase in tumor-free survival and a lesser mean tumor volume was noted, these effects were not statistically significant (data not shown). Palpable tumors started to develop 8 days after tumor challenge in naïve animals. When a dose of $3x10^{10}$ particles of rAAV-sFlt1 was given, tumor-free survival was seen in

>33% of the animals ($p>0.05$) whereas a dose of $3x10^{11}$ particles protected 83% of the mice ($p<0.005$). At a higher vector dose, there was also a significant difference in the mean tumor volume between different treatments (naïve or GFP versus sFlt-1, $p<0.0001$). There was a slight delay in the appearance of tumors in the rAAV-GFP treated group compared to naive animals, which did not receive any vector.

rAAV-sFlt-1 therapy does not cause liver damage. Previous studies have indicated that higher levels of sFlt-1 in mouse liver cause significant hepatotoxicity associated with hemorrhage of blood vessels in the liver (14). VEGF has been reported to regulate the proliferation and survival of the sinusoidal endothelial cells acting through the VEGF receptors Flt-1 and Flk-1 (26,27). Blockade of the receptors, resulting from higher concentrations of sFlt-1 leads to such damage. Thus overexpression of sFlt-1 from systemically delivered adenovirus vector, which endogenously targets liver, has been reported to result in similar damage (14). To determine if stable systemic expression of sFlt-1 by rAAV leads to liver toxicity, liver tissues were macroscopically and microscopically examined following rAAV-sFlt-1 treatment. Hematoxylin and eosin (H&E) stained sections from naïve and AAV-sFlt-1 treated mice showed no cytomorphological signs of hepatotoxicity strongly suggesting that systemic expression of sFlt-1 from skeletal muscle does not result in liver toxicity (Figure 7). Further, immunohistochemical staining for human sFlt-1 in the liver sections did not reveal accumulation of systemically expressed sFlt-1 (data not shown)

indicating that delivery of rAAV-sFlt-1 in skeletal muscle and systemic secretion of the factor at stable levels would be advantageous. Also, several studies using rAAV encoding a variety of therapeutic proteins have shown stable systemic expression of transgenic factors following intramuscular vector administration. No apparent effects such as loss of body weight, general mobility or food uptake was observed in the rAAV-sFlt-1 treated mice compared to untreated mice that were not tumor challenged.

Discussion

Recent studies have indicated the potential of sFlt-1 gene therapy in murine models of human diseases using viral vectors (15,28-32). Whereas studies using adenoviral and retroviral vectors have reported the efficacy of sFlt-1 gene therapy in inhibiting the growth of human tumors, that using AAV reported the effects in retinal diseases and *in vivo* growth of a stably transduced cell line (31,32). The present study demonstrates that stable expression of sFlt-1 as a secretory protein from skeletal muscle by rAAV provides significant protection and long-term survival of mice against the growth of a human tumor xenograft suggesting its potential in cancer gene therapy.

The potential use of rAAV-sFlt1 as a vector for anti-angiogenic therapy of solid tumors is multifold. Since tumor angiogenesis is mediated by a cascade of signals provided by cells of both tumor and endothelial origin, which ultimately results in the growth and differentiation of endothelial cells forming the tumor neovasculature, therapy targeting such an event should be sustained to effect a maximal tumoristatic response. Stable expression of transgenic factors as secretory proteins by administering rAAV in skeletal muscle has been well established in preclinical models (6,7,33-37) and formed the basis for a human clinical trial (38). Thus, the proven efficiency of muscle-based administration of rAAV-sFlt-1 should provide sustained anti-angiogenic effects. Since sFlt-1 is a native protein, there is no expected host immune response against the transgenic factor when used for therapy in humans. Further, the cytolytic T-cell response against rAAV-transduced muscle is known to be minimal due to both

low-immunogenicity against the vector and poor transduction of rAAV to dendritic cells (24). Thus, the potential of muscle-based rAAV-sFlt-1 therapy should be advantageous. Although several drugs have shown promise in controlling tumor neovasculature, a major problem in pharmacotherapy is the profound side effects of constant administration due to their limited half-life (39). Gene therapy, on the other hand, offers advantages of maximizing cost effectiveness while maintaining sustained levels of anti-angiogenic factors.

Although many factors are known to play important roles in new blood vessel formation, a key molecule promoting the growth of tumor neovasculature is VEGF, which has been considered a predictive marker in many human cancers (9-13). Further, overexpression of VEGF mRNA and elevated serum VEGF levels have been correlated with decreased survival in many neoplastic conditions including ovarian cancer (40). Despite higher levels of plasma VEGF levels observed in many human cancers, there was no concomitant increase in sFlt-1 levels in cancer patients indicating an imbalance in the native anti-angiogenic signal pathway (41). In patients who showed earlier relapse of breast cancer, tumor VEGF levels were higher than in patients with a longer disease-free survival and the rate of response to chemotherapy decreased with higher VEGF levels (42). Hence, a stable gene therapy approach targeting VEGF as an adjuvant therapy offers the promise of increased survival in patients.

Inhibition of endogenous VEGF levels by the administration of VEGF antibody alone or in combination of topoisomerase inhibitors has resulted in effective immunotherapy and reduction of Wilms' tumor respectively in murine

models (43,44). Unlike other biologically-driven anti-angiogenic factors such as angiostatin and endostatin whose mechanism of action are not fully elucidated despite their anti-angiogenic effects, the biological properties of sFlt-1 are well known. The sFlt-1 acts by both sequestering VEGF and blocking VEGF receptors from binding to VEGF (8). Thus, therapy targeting VEGF will have specific effects on tumor growth inhibition and metastasis.

We have recently reported that intravenous administration of adenovirus encoding sFlt-1 results in systemic toxicity due to high sFlt-1 expression in liver (14). By using an adenovirus encoding GFP, the toxicity was determined to be due to increased sFlt-1 levels rather than vector localization in liver. Thus, using skeletal muscle as a platform to achieve systemically therapeutic levels of sFlt-1, rAAV can overcome such a deleterious effect in addition to offering the advantage of long-term expression. In a recent preclinical study, we also demonstrated that significant inhibition of tumor growth can be achieved by intramuscular administration of rAAV encoding angiostatin and endostatin as secretory proteins (45). Histopathological analysis of liver from rAAV-sFlt-1 treated mice liver also confirmed the absence of any cytomorphic damage.

Results of our studies additionally indicated that significant therapeutic effect was seen only with a vector dose of $3x10^{11}$ particles in mice. Thus, a clinical translation of these findings would require careful determination of optimal vector dose. Although we did not quantitatively determine the systemic levels of sFlt-1 following therapy, at the therapeutically efficacious dose, immunohistochemical analysis of rAAV-sFlt-1 injected muscle and adjacent blood

vessels clearly indicated abundant expression and secretion of sFlt-1 suggesting that delivery of the sFlt-1 transgene in muscle is a safer method of overcoming any potential toxicity due to systemic delivery of a recombinant adenovirus encoding sFlt-1.

A recent study on the potential use of rAAV encoding sFlt-1 in ovarian cancer reported that transduction of the human ovarian cancer cell line, RMG-1, with AAV-sFlt1 *in vitro* followed by intraperitoneal administration in nude mice resulted in a decrease in proliferative and metastatic indices, further suggesting the feasibility of localized AAV-sFlt-1 anti-angiogenic gene therapy (32). However, a major limitation of intratumoral delivery is the limited transduction efficiency and dispersion of the vector within the tumor cells. Also, unlike certain genetic metabolic diseases, which require only partial amounts of the deficient protein/enzyme for phenotypic correction of the disease, tumor therapy requires inhibition of the tumor growth *in toto*. Anti-angiogenic therapy, in particular, requires a constant level of the inhibitory factor(s) for a sustained therapeutic effect. Thus, the strategy that we adapted in the present study will likely have greater potential and translational utility for human cancers, in particular, as an adjuvant therapy against tumor recurrence. Although several preclinical studies have shown that stable levels of systemically secreted proteins using rAAV resulted in the phenotypic correction of inherited metabolic defects (6,7), accumulation of anti-angiogenic factors in other organs due to unregulated expression may lead to ischemic conditions or impair wound healing. Thus, future studies are warranted to test the efficacy of regulated expression of these

factors by using inducible promoters (46,47) for a safe muscle-based rAAV anti-angiogenic gene therapy.

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Figure Legends

Figure 1. Recombinant AAV encoding human sFlt-1. rAAV containing human sFlt1 was subcloned under the control of the CMV promoter. Poly A represents the SV40 late polyadenylation signal sequence and ITR represents the inverted terminal repeat sequence of AAV.

Figure 2. Western blot analysis of rAAV-sFlt-1 expression. 293 cells were either mock-transduced (2) or transduced with rAAV encoding sFlt-1 (3). Forty-eight hrs after transduction, the supernatant was analyzed by Western blotting using a monoclonal antibody against human sFlt-1. A recombinant purified sFlt-1 protein, fused to the Fc-portion of immunoglobulin was used as a positive control (lane 1). A band of approximately 50 kD, from non-specific hybridization is seen in both mock-transduced and rAAV-sFlt-1 transduced lanes.

Figure 3. Endothelial cell proliferation assay to determine the biological activity of rAAV-sFlt-1. rAAV encoding sFlt-1 was transduced into 293 cells at an MOI of 100. Forty-eight hours later, 25 µl of supernatant from mock-transduced (A) or rAAV-sFlt-1transduced (B) cells were tested on HUVEC. Cell proliferation was determined by fixing and staining the HUVEC with crystal violet. Purified recombinant human angiostatin was used at a concentration of 20 µg/ml (C) as a positive control. Magnification x20.

Figure 4. PCR, *In situ* hybridization and immunohistochemistry for long-term transgene persistence and expression. Top Pane: PCR was performed using genomic DNA isolated from naïve or rAAV-sFlt-1 injected muscle using a primer pair specific for the amplification of the transgene. Whereas no amplification product was seen in DNA from naïve mice, (lanes 1 & 2), a 548 bp fragment, specific for the transgene, was amplified from rAAV-sFlt-1 treated mice [lanes 3 & 4, (i)]. Sham- or rAAV-transduced muscle tissues were harvested and immediately fixed in buffered-formalin for 24 hr and then embedded in paraffin. *In situ* hybridization was performed using a DIG-labeled DNA probe on sections obtained from naïve (ii), or rAAV-sFlt1 (iii) administered muscle tissues. A two-fold magnification of a specific area is indicated in the box, showing positive signal only in the vector-transduced group. **Bottom Panel:** Immunohistochemistry was performed using a human VEGF receptor-1 antibody, which recognizes the extracellular domain of VEGF receptor. Extracellular secretion of the transgenic factor from the vector-transduced muscle tissue is evident in rAAV-sFlt1 treated animal (B) compared to naïve mouse (A) .

Figure 5. Growth characteristics of subcutaneously implanted SKOV3.ip1 cells in athymic nude mice following therapy with rAAV-sFlt-1. Three weeks after sham (♦), $3x10^{11}$ AAV-GFP (□), or rAAV-sFlt1 (▲) injection, 10^7 SKOV3.ip1 cells were subcutaneously implanted. Tumor size was measured using a digital caliper. The average tumor volume in each group is given in mm^3. * indicates *P* value <0.0001, compared to naïve and AAV-GFP groups.

Figure 6. Tumor-free survival of mice following rAAV-sFlt-1 therapy. $3x10^{11}$ particles of rAAV encoding GFP, or sFlt1 were injected into the quadriceps muscle in the hind limb of athymic nude mice. Three weeks after the vector injection, the animals were challenged with 10^7 SKOV3.ip1 cells in each flank subcutaneously and tumor-free survival recorded. The mean tumor-free survival of naïve (Δ), rAAV-GFP (□), or rAAV-sFlt1 (●) is given above.

Figure 7. Absence of hepatotoxicity following rAAV-sFlt-1 therapy. Livers from naïve or rAAV-sFlt-1 treated mice were formalin-fixed, sectioned to 5 μm thickness and stained with H&E. The stained slides were analyzed by light microscopy for hepatotoxicity including hemorrhage and congestion, focal necrosis with minimal inflammation, hepatocyte dropout, or increased apoptosis. There was no difference that existed in the histopathology of liver sections between naïve mice (A) and rAAV-sFlt1-treated mice (B).

Figure 1

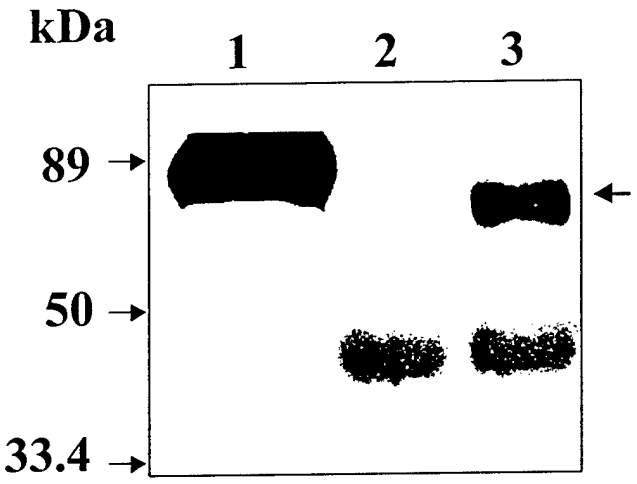


Figure 2

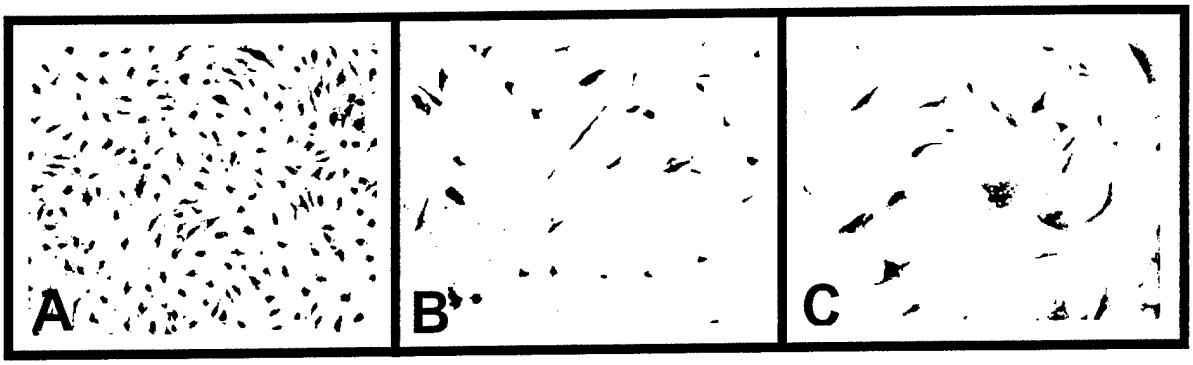


Figure 3

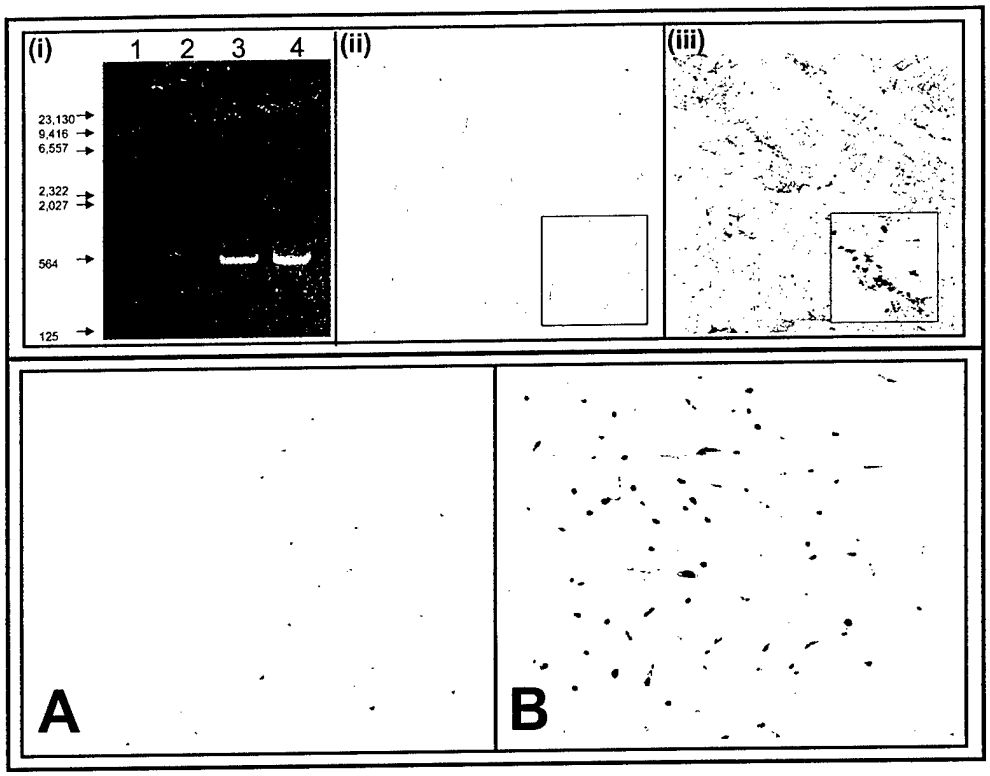
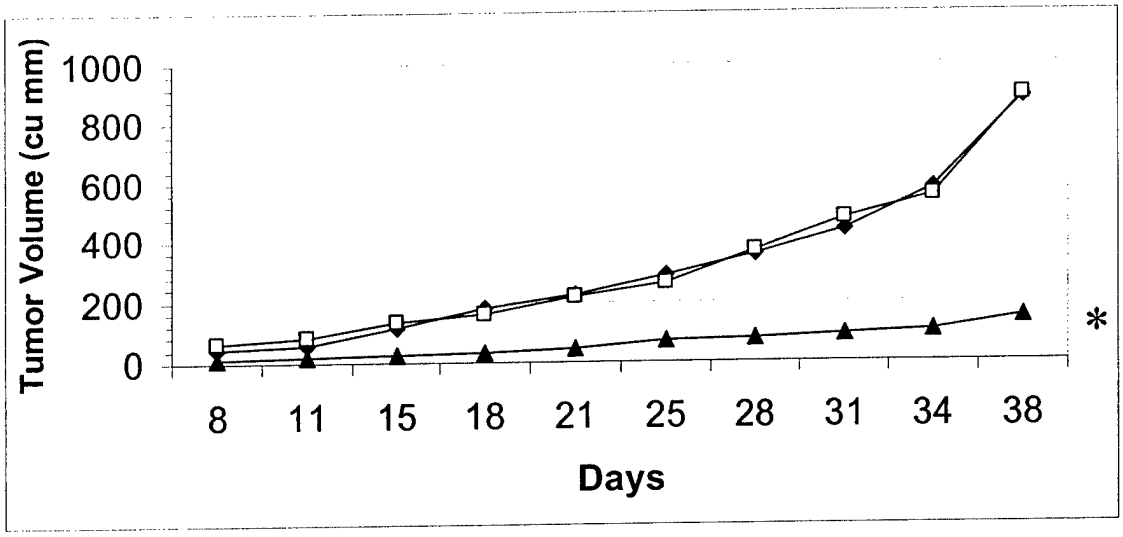


Figure 4


Tumor Volume (cu mm)
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8
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Days
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Figure 5

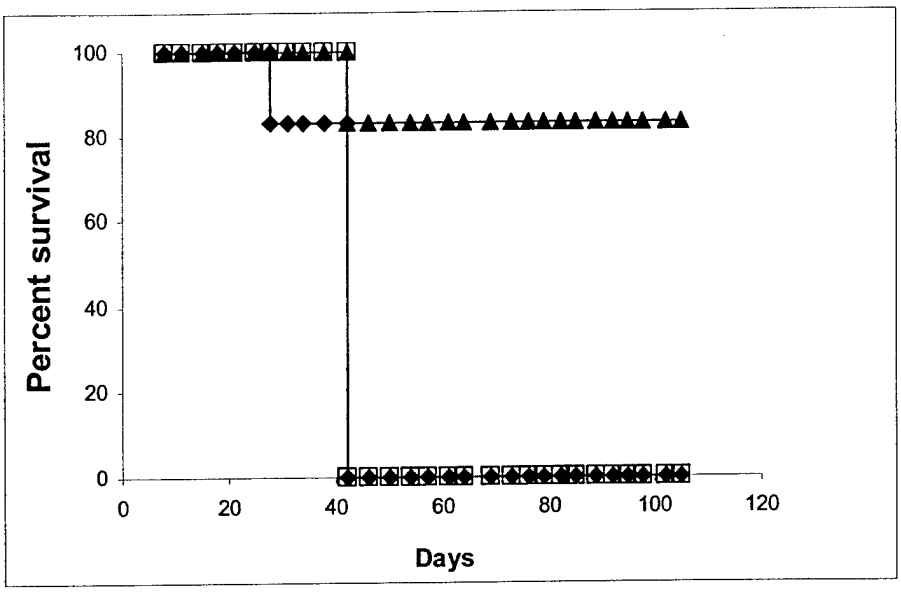

Figure 6

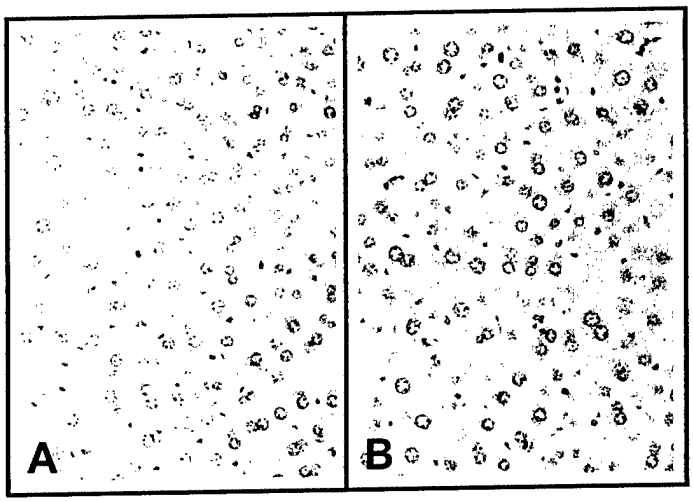

Figure 7

Appendix 3

Anti-angiogenic gene therapy for cancer (Review)

TATYANA ISAYEVA, SANJAY KUMAR and SELVARANGAN PONNAZHAGAN

Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract. Angiogenesis is a prerequisite for the growth and metastasis of solid tumors. Studies have confirmed that in the absence of angiogenesis, tumors rarely have the ability to develop beyond a few millimeters in diameter. Tumor-associated endothelium is activated by the production of soluble factors by tumor and stromal cells. Tumor-associated endothelial cells are physiologically homogeneous and divide more frequently. Thus, targeting the proliferation of tumor neovasculature will form an effective anti-cancer therapy. With the identification of drug and biological molecules that inhibit the growth of tumor endothelium, several attempts have been made in preclinical and clinical research to evaluate the potential of anti-angiogenic therapy. Although several drugs and purified proteins have shown promise, their widespread application is limited by half-life, side effects and cost involved. Gene therapy approaches, on the other hand, have the potential to overcome these limitations. Further, genetic transfer of anti-angiogenic genes can be combined with other treatments including radiation therapy or chemotherapy for synergistic effects. In this review, we provide a comprehensive account of factors and mechanisms underlying tumor angiogenesis, the potential and limitations of available gene therapy vectors for anti-angiogenesis and current status of preclinical and clinical gene therapy studies targeting tumor angiogenesis.

Contents

1. Introduction
2. Major therapies for cancer management
3. Alternate therapies for cancer
4. Potential anti-angiogenic factors for gene therapy
5. Vectors for anti-angiogenic gene therapy
6. Clinical trials targeting tumor angiogenesis

Correspondence to: Dr Selvarangan Ponnazhagan, Department of Pathology, University of Alabama at Birmingham, LHRB 513, 701, 19th Street South, Birmingham, AL 35294-0007, USA
E-mail: sponnazh@path.uab.edu

Key words: angiogenesis, endothelial cells

1. Introduction

Angiogenesis is the process of new capillary blood vessel formation from pre-existing vessels (1). Angiogenesis is a complex process encompassing a number of overlapping phases of activation, migration and proliferation of endothelial cells; digestion of basement membrane and underlying stroma, ultimately forming new networks of blood vessels (2-5). In normal situation, the process of angiogenesis plays a key role in female reproductive cycle, wound healing, and embryonic development (6). Most of the time, endothelial cells remain dormant or rarely divide, doing so only about once every 3 years on average (7). However, when capillary growth is uncontrolled, angiogenesis may become pathological and the resulting excessive neovascularization may then sustain the development of diseases including retinopathies, hemangiomas, rheumatoid arthritis, psoriasis, vascular diseases and solid tumor growth (8,9). The present article will focus on the mechanism, implications and possible intervention of tumor angiogenesis with emphasis on gene-based therapy for cancer.

2. Major therapies for cancer management

Accumulating results of many observations indicate that tumor growth and metastasis is dependent on angiogenesis, and that solid tumors cannot grow beyond the size of 1-2 mm^2 without inducing the formation of new blood vessels to supply the nutritional needs of the tumor (10). Metastatic spread of solid tumors also depends on vascularization of the primary mass (11). Depending upon the stage of diagnosis of solid tumors, major forms of therapeutic intervention are surgery, chemotherapy and radiotherapy. Surgery is one of the main treatments for cancer. This strategy is used to remove the tumor and nearby tissues that might contain cancer cells. However, if the cancer has spread and cannot be totally removed by surgery, surgery is used to remove only the primary tumor (12,13).

Chemotherapy is a general term that is used to describe killing of cancer cells by drugs. Chemotherapy can be used as a primary therapy or as an additional therapy following surgery and/or radiation therapy (14). Many such drugs destroy cancer cells by preventing them from growing and dividing rapidly. Unfortunately, a number of the body's normal non-cancerous cells also divide rapidly and therefore are harmed by chemotherapy (15-18). The most common chemotherapeutic agents are the nitrosoureas [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)], platinum-based drugs (cisplatin, cisplatinum, carbo-

platin), temozolomide, procarbazine, and natural-occurring compounds (taxol) (19,20). The drawbacks to this therapy are that not all tumors are responsive to chemotherapeutic drugs, and treatment causes damage to the bone marrow of the patient. Actively dividing cells are most susceptible to this form of treatment. The dose, timing, and choice of chemotherapies are determined by factors such as the patient's type and stage of cancer, response to and recovery from chemotherapy, and health status. Different types of chemotherapeutic agents affect different phases of the cancer cell cycle. A combination of chemotherapeutic drugs helps to increase the cancer-fighting potential of treatment and also helps to keep cancer cells from becoming resistant to individual drugs (21).

Radiotherapy is used to kill cancer cells by exposure to high-energy radiation. Radiotherapy is a localized treatment, which can reduce the recurrence of tumor after surgery (22,23). Cancer cells are more sensitive to radiotherapy than normal cells. Chemotherapy and radiation have the disadvantage of destroying normal as well as malignant cells and thus can cause severe side effects (24,25). Further, chemotherapy and radiation resistant tumor cells cause recurrence and uncontrolled metastasis. These limitations underline the importance of development of other forms of cancer treatment.

3. Alternate therapies for cancer

The alternate forms of cancer therapy with great potential are immunotherapy, radioimmunotherapy and gene therapy. Combination of all foregoing methods represents a new and powerful tactics in the arsenal of anti-cancer treatments. Immunotherapy involves the use of cytokines and antibodies in order to mobilize the body's immune defenses against cancer cells (26-28). Typically, active cancer immunotherapy aims to modulate the function of tumor-specific cytolytic T-cells (CTLs) and antigen presenting cells (APCs), especially dendritic cells (DCs) that play a critical role in both innate and adaptive immune responses. This includes enhancing the function of APCs by promoting the growth and differentiation of DCs, potentiating T cell activation by improving co-stimulation and engineering vaccine vectors to significantly enhance the immunogenicity of vaccinogens (29,30). Radioimmunotherapy (RIT) strategy is aimed at destroying tumor cells by the radiation from the radio-labeled monoclonal antibodies targeted to tumor-specific antigens (31,32). Ideally, all anti-bodies, used for immunotherapy and RIT would be targeted to molecules expressed on the cancer cells but not on normal cells (33,34).

In a simpler sense, gene therapy can be described as the process of transferring genetic materials (DNA or RNA) to modify the genetic repertoire of targeted cells for therapeutic application (23). Gene therapy, conformably to neoplastic diseases, is gene transfer to cancer cells or tumor microenvironment in order to obtain direct or indirect therapeutic (damaging) effects to cancer cells. After extensive investigation in preclinical studies and recent clinical trials, gene therapy has been established as a potential method for cancer therapy (35-39).

Gene therapy strategies encompass a wide spectrum of therapeutic intervention including immunotherapy, anti-angiogenesis, cytotoxic gene transfer, restoration tumor suppression function. Many of the genes that are upregulated by aggressive cancer cells include those that are involved in angiogenesis and vasculogenesis. A highly vascularized tumor is correlated with a poor clinical prognosis not only because of the potential for uncontrolled tumor growth, but also because of increased access of the tumor to capillaries, which contribute to increased metastatic potential (40).

4. Potential anti-angiogenic factors for gene therapy

Induction of the angiogenic switch depends on a local change in the balance between activators and inhibitors of angiogenesis (41). The switch to an angiogenic phenotype of tumor requires upregulation of angiogenic factors and down-regulation of angiogenesis inhibitors (9,42,43). Pro-angiogenic gene expression is increased by physiological stimuli, such as hypoxia via the hypoxia-inducible transcription factors HIF-ß HIF-1α, and HIF-2α, which results from increased tissue mass, and also by oncogene activation or tumor-suppressor mutation (44-46). Once the tumor becomes angiogenic, tumor-infiltrating endothelial cells and stromal cells produce tumor growth stimulators (47,48). Tumor cells and macrophages recruited into the tumor are able to release high levels of angiogenic factors, which can activate a cascade of angiogenesis mediators. Until now, many angiogenic peptides have been identified, including vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF) (49,50), basic and acidic fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), interleukin-8 (IL-8), transforming growth factor-α and ß (TGF-α and -ß), heparin growth factor, granulocyte colony stimulating factor, E-selectin, angiogenin, tumor necrosis factor-α (TNF-α) (51-53). Angiogenic factors released by tumor cells stimulate endothelial cells (EC) which become activated, providing growth factors (FGF-1, -2) and matrix metalloproteinases (MMPs) (54). Proteolytic activities of the MMPs degrade the surrounding tissue and vascular membrane, leading to EC proliferation, migration and new capillary formation (Fig. 1) (55,56).

Thus, the goal of anti-angiogenic gene therapy is to switch the local balance between angiogenic factors and angiogenic inhibitors in tumor microenvironment to the anti-angiogenic phenotype, targeting one or more events of angiogenesis cascade. Anti-angiogenic factors can regulate tumor activity at different levels of angiogenesis. The more promising angiosuppressive agents for clinical testing are: naturally occurring inhibitors of angiogenesis including angiostatin, endostatin, platelet factor-4 (57-65), specific inhibitors of endothelial cell growth including TNP-470, thalidomide, interleukin-12 (66-68), agents neutralizing angiogenic peptides such as antibodies to fibroblast growth factor or vascular endothelial growth factor, suramin and analogs, tecogalan and others (69) or their receptors, agents that interfere with vascular basement membrane and extracellular matrix MMP inhibitors, angiostatic steroids and others, anti-adhesion molecules antibodies such as anti-integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, and miscellaneous drugs that modulate angiogenesis by diverse mechanisms of action (Fig. 1) (70-72).

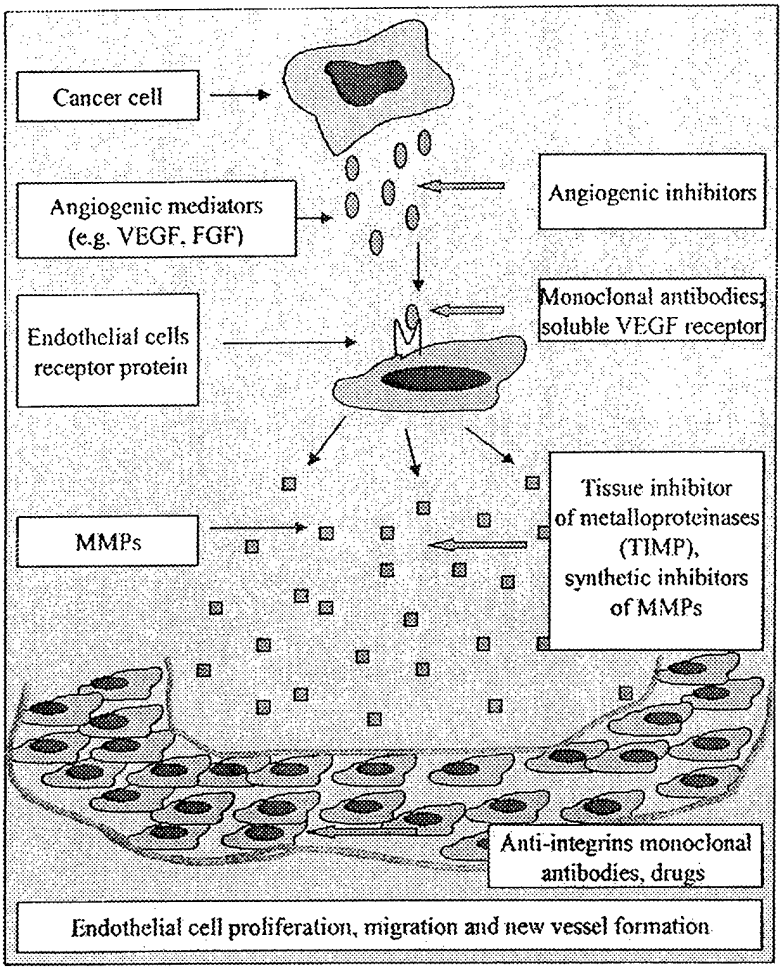

Figure 1. Tumor angiogenic signaling cascade and target pathways of therapeutic inhibitors. Factors and cells that promote tumor angiogenesis are indicated in the left boxes whereas potential inhibitors for each stage of angiogenic cascade are indicated in boxes on the right. Upregulation of endothelial growth factors by the tumor cells promote a series of events leading to activation, proliferation and migration of endothelial cells which eventually invade degraded matrix to form new blood vessels. Therapy targeting this cascade can be directed at either one or more events.

5. Vectors for anti-angiogenic gene therapy

Gene transfer to target cells is accomplished with gene carriers commonly termed 'vectors'. The design of a vector should allow efficient transfer of therapeutic gene to targeted cells and effectively translocating it to the site of expression. Typically, genes to be transferred are in the form of complementary DNA (cDNA) coding for a therapeutic protein. Therapeutic genes are contained in 'expression cassette' that includes a constitutive or regulatable promoter for the cDNA expression. Several strategies are used in gene therapy for delivering gene of interest *in vivo* for modulation of neovascularization. These can be broadly classified in to viral or non-viral vectors (73,74).

Viral vectors. Viral vectors have the ability to transduce cells through specific receptors and effectively transfer their genome to a functional site, where the host cell machinery will drive the expression cassette for the production of transgenic protein. There is no single viral vector, which can suit all gene therapy approaches. Hence, each clinical approach will require appropriate modification of viral vectors. To effectively utilize a virus as a gene transfer vector, sufficient viral genes are removed to permit insertion of an expression cassette and also to assume the non-toxic virus a replication deficient one. Strategy of viral gene transfer can be *ex vivo* or *in vivo*. *Ex vivo* approach requires target cells to be isolated, purification of the gene and transfer is performed *in vitro* in laboratory. Genetically altered cells are then transferred back to recipient in the region of the tumor growth. Although the *ex vivo* approach gives control for the target cells, it is labor intensive and expensive. *In vivo* gene transfer mostly targets specific organs *in situ* for concentrated and feasible therapeutic gene expression. This approach limits the area by degree of certainty in determining which specific cells are affected by gene transfer.

Retroviral vectors (RNA viruses) were one of the first vectors developed for use in gene therapy. They exist as lipid-enveloped nucleoprotein particles containing a single-stranded linear RNA molecule of about 7 kb. Retroviral infection is initiated by attachment to the retroviral cell surface receptors (75,76). On entry into cytoplasm, RNA is reverse-transcribed into DNA and then randomly integrated into the host cell genome. Wild-type retroviruses tend to establish chronic non-pathological infections, but can also cause malignancy and immunodeficiency states. Most recombinant retroviral vectors

Table I. Recombinant viral vectors and therapeutic genes used in preclinical efficacy of anti-angiogenic therapy for cancer.

Vector	Therapeutic molecules	Tumor type	Delivery route	Author/Refs.
Vesicular stomatitis virus-pseudotyped lentiviral vectors	Angiopoietin receptor Tie2/Tek	s.c. (TS/A)	i.v.	De Palma *et al* (111)
Recombinant adeno-virus vector	Antisense uPAR; antisense	Intracranial (SNB19)	*ex vivo* transduction and s.c.	Lakka *et al* (112) Liu *et al* (113)
	MMP-9; TSP-1	s.c. (K562)	i.t.	Maemondo *et al* (114) Hoshida *et al* (115)
	NK4	s.c. (A549; H358)	*ex vivo* transduction	Lefesvre *et al* (116)
	The soluble form of flt-1 VEGF receptor	s.c. (Panc-1; PK-8) s.c. and tail vein	i.t. i.v. or i.t.	
	ATF-BPTI	(L42 and L44)		
Cationic vector (GL67/ DOPE or PEI22K)	Endostatin	i.v. (NFSa Y83)	i.v.	Nakashima *et al* (117)
Eukaryotic expression vector pcDNA3.1(+)	Angiostatin	s.c. (SGC7901)	*ex vivo* transfection	Wu *et al* (118)
Aden-associated virus vector	KDR	s.c. (EJ cells)	*ex vivo* transduction	Zhang *et al* (119) Davidoff *et al* (120)
	Flk-1	s.c.; into the kidneys and i.t (SK-NEP-1)	intraportal	Xu *et al* (121) Shi *et al* (122)
	Angiostatin	into the liver (EL-4)	intraportal	Shi *et al* (123)
	Endostatin	i.m. (HT29)	i.m.	
Retroviral vector	Endostatin	s.c. (SMMC7721)	*ex vivo* transduction	Wang *et al* (124)
	Endostatin	s.c. or i.p. (NMuLi) i.c. (B16 melanoma)	*ex vivo* transduction	Feldman *et al* (125) Zhang *et al* (126) Indraccolo *et al* (127)
	Endostatin	s.c. (MCF7; MDA-MB435 breast cancer cells)	i.t.	
	Angiostatin, endostatin and interferon-α		*ex vivo* transduction	
Lentiviral vector	Non-catalytic fragment of MMP2 (PEX)	s.c. (B16 melanoma)	i.t.	Pfeifer *et al* (128)

are based on the murine leukemia virus. They are able to target dividing cells with a high degree of efficiency and a moderate level of gene expression. Retrovirus vectors are most useful in *ex vivo* gene transfer strategy but their *in vivo* application has limitation since, they need proliferating cells for transduction (77).

Lentiviruses are a subclass of retroviruses. The lentiviral vectors derived from the human immunodeficiency virus (HIV) and are being evaluated for safety, with a view to removing some of the non-essential regulatory genes. Unlike the retroviruses, the lentivirus vectors can infect non-dividing or terminally differentiated cells such as neurons, macrophages, hematopoietic stem cells, retinal photoreceptors, and muscle and liver cells, cell types for which previous gene therapy methods could not be used (78-80). The main limitation of the use of retroviruses and lentiviral vectors concerns the issue of safety. It has been shown that recombination can occur in producer cell lines leading to the production of replication competent viruses and potential infection in the host. Retroviral DNA integrates randomly into the host genome and this

ability might lead to insertional mutagenesis of a cellular oncogene leading to tumor formation (81). Using retroviral/lentiviral vectors transfer of endostatin, angiostatin and INF-α in murine models have shown therapeutic efficacy against the growth of human liver carcinoma cell, breast cancer and melanoma cells (Table I).

Adenoviral vectors are based on a family of viruses that cause benign respiratory tract infections in humans and there are about 42 serotypes of adenovirus known to infect humans. Adenoviral vectors used in gene therapy are typically based on serotype 5, with the majority of the E1a and E1b regions deleted to prevent virus replication. The E3 region can also be deleted to provide additional space for the insertions of up to 7.5 kb of exogenous DNA. The adenoviral genome enters dividing, as well as non-dividing cells by receptor-mediated endocytosis. Infection is initiated by the binding of the fiber protein to the coxsackie virus and adenovirus receptor (CAR), which is expressed at the cell surface (82,83). Disadvantage of adenoviral vectors is the relative lack of specificity with which they transduce the cells. The adenovirus receptor is virtually ubiquitous and consequently systemic administration is likely to lead to adenoviral uptake in cell types other than the target cell thereby reducing the specificity of the gene therapy. Replication deficient recombinant adenovirus (rAd) vectors are easy to produce in large amounts with high degree of purity and transfers genes to all cell type regardless of state of differentiation of cell type, resulting in robust expression of transduced genes. However, they do not integrate into the genome so, expression is for a short period of time lasting from days to weeks or months. Evaluation of the contribution of the innate immune component of host defense in clearing the genome of adenovirus vectors following *in vivo* administration have shown, that 90% of vector DNA is eliminated within 24 h and degraded in the liver by a non-immune-mediated mechanism following intravenous delivery (84). The immune response elicited by these vectors *in vivo* is probably one of their major drawbacks. Most adults have had prior exposure to wild-type adenovirus and, therefore, possess anti-Ad antibodies. Studies show that 55% of adults have antibodies capable of neutralizing the infection of Ad5, the most widely used serotype (85).

For anti-angiogenic purpose recombinant adenovirus was successfully used for *in vivo* delivery and expression several anti-angiogenic factors including antisense urokinase plasminogen activator receptor, antisense MMP-9, thrombospondin-1, hepatocyte growth factor (Table I).

Adeno-associated virus (AAV) is a small single-stranded DNA containing non enveloped parvovirus that is able to integrate into the host genome during replication, thereby producing stable transduction of the target cell. The virus can also infect a wide range of cell types, including dividing and non-dividing cells (86). For productive infection to occur, AAV requires coinfection with a helper virus, which allows the viral genome to replicate episomally, and leads to synthesis of AAV proteins (87). In the absence of helper virus, the wild-type AAV genome can integrate into the host cell chromosome where it remains in a latent state until infection with a helper virus occurs. The integration is preferential (about 70%) to a site on chromosome 19 (19q13.3-qter). Site-specific integration requires enzyme activity of replication (Rep) proteins (Rep68 and/or Rep78) (88-91). Recombinant AAV are devoid of wild-type viral genes, hence, site specificity is lost during limited integration of recombinant AAV. Recombinant AAV (rAAV) is developed by deleting the entire coding region. Production of rAAV is achieved by transcomplementation of rep and capsid (cap) genes through helper plasmids in transient packaging cells (92). AAV-based vectors are becoming increasingly popular for gene therapy of human diseases (93,94). Despite the potential for long-term expression, genomic integration, and low immunogenicity, the transduction efficiency of AAV type 2 (AAV-2) vectors varies significantly among cell types: neurons, liver (95), muscle (96), and airway epithelium (97). Recent studies using AAV vectors have shown therapeutic efficiency following the gene transfer of extracellular domain of kinase domain region, a soluble, truncated form of the vascular endothelial growth factor receptor-2, angiostatin, endostatin in animal models (Table I). A disadvantage with rAAV is limited accommodative capacity for the size of expression cassette (around 4.5 kb) and like other viruses they elicit humoral immune response against capsid protein, which may limit the readmistration of the AAV vectors (94).

The limitations of viral vectors, in particular their safety concerns, difficulty in targeting to specific cell types have led to the evaluation and development of alternative vectors based on synthetic, non-viral systems.

Non-viral vectors. The main alternatives to viruses for gene transfer include liposomes, naked DNA, liposome/polycation/DNA (LPD) complexes and peptide delivery systems (98). Negatively-charged liposomes have been used to deliver encapsulated drugs for some time and have also been used as vehicles for gene transfer into cells in culture. Problems with the efficiency of nucleic acid encapsulation, coupled with a requirement to separate the DNA-liposome complexes from 'ghost' vesicles has lead to the development of positively-charged liposomes. Cationic lipids are able to interact spontaneously with negatively-charged DNA to form clusters of aggregated vesicles along the nucleic acid (99).

In animal models studies have shown inhibition of lung tumor angiogenesis by production of endostatin gene complexed with a cationic vector (GL67/DOPE or PEI22K) (Table I). The simplest non-viral gene delivery system uses naked expression vector DNA. Direct injection of free DNA into certain tissues, particularly muscle, has been shown to produce surprisingly high levels of gene expression, and the simplicity of this approach has led to its adoption in a number of clinical protocols (100,101). The limitations of liposome mediated gene delivery have led to the development of novel lipids in an effort to improve these vectors. This method involves polycations (such as polylysine or protamine) in the formation of liposome/DNA complexes. These complexes have shown higher transfection efficiency than liposome/DNA complexes without the presence of polycations and also showed enhanced resistance to degradation by nucleases.

Another type of non-viral vector is designed to contain an expression cassette on a plasmid backbone. Practical advantages of non-viral vectors include their ability to accommodate larger expression cassettes and they do not

elicit vigorous host immune response. But the use of plasmid vectors has limitations such as low gene transfer efficacy and transient transgene expression, hence, may need repeated administration. This has resulted in low levels of their use in clinical trials (102,103). However, the non-viral gene transfer technology is relatively inexpensive and easy to handle.

6. Clinical trials targeting tumor angiogenesis

Tumor angiogenic therapy using gene transfer vector widens the approach by its ability to produce the therapeutic molecules at high concentration within the localized target cells for a sustained period of time with a single administration (using gene transfer vectors for anti-angiogenesis therapy fulfills many of these requirements).

Endostatin is the first endogenous angiogenesis inhibitor to enter clinical trials. Final results from the phase I trial of endostatin at The University of Texas M.D. Anderson Cancer Center show that the anti-angiogenic drug was safe and reduced blood flow in patients. There was some shrinkage of tumors among the participants, and two patients showed encouraging results (104).

Endostatin is a 20-kDa proteolytic COOH-terminal fragment derived from collagen XVIII (58). Endostatin reduces endothelial cell proliferation and migration, significantly reduced invasion of endothelial as well as tumor cells into the reconstituted basement membrane and acts as a potent inhibitor of angiogenesis and tumor growth. Endostatin inhibits the proteolytic activation of proMMP-2 and the catalytic activities of membrane-type 1-MMP and MMP-2. In pre-clinical studies, expression of endostatin decreased tumor vascularization 3-fold with a concomitant 3-fold increase in the number of apoptotic cells (105).

Another potent angiogenesis inhibitor is angiostatin (38 kDa), which is generated as a result of proteolytic cleavage of plasminogen and comprises the first four triple loop disulphide-linked structures of plasminogen, termed kringle domains (106). Plasminogen is anticoagulant and does not have anti-angiogenic activity. Analysis of the activity of kringle (K) fragments of angiostatin (K1-4) demonstrated that K1, K2 and K3, inhibited endothelial cell proliferation, whereas a structure combining K1-3 was more effective than K1-4. The K5 proteolytic fragment of human plasminogen is an even more potent inhibitor of endothelial proliferation. Urokinase-activated plasmin can also convert plasminogen into a molecule containing the intact K1-4 and most of the K5 domains, termed K1-5. K1-5 inhibited endothelial cell proliferation more effectively than did angiostatin, moreover, K1-5 treatment suppressed tumor growth and neovascularization in mice carrying a primary fibrosarcoma (107,108). Currently, phase II/III clinical trials of endostatin and angiostatin are ongoing, and preliminary data analysis show minimal toxicities but anti-tumor results are inconsistent (109,110).

Thus, a combination of identification of most potent anti-angiogenesis factors in actual cancer patients and optimization of vector strategies to achieve persistent transgene expression targeting tumor neovascularization without interfering with the normal cells should lead to a wide-spread application of this technology in future. Further, anti-angiogenic gene therapy also can be used as an effective combination therapy, for example, with chemotherapy, radiotherapy or surgery, to enhance the period of remission, against recurrence and metastasis.

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Appendix 4

Recombinant adeno-associated virus 2-mediated anti-angiogenic gene therapy in a mouse model of intraperitoneal ovarian cancer

(Running Title: Gene therapy for intraperitoneal ovarian cancer)

Tatyana Isayeva, Changchun Ren, and **Selvarangan Ponnazhagan**

Department of Pathology

The University of Alabama at Birmingham

Birmingham, AL 35294

*Corresponding author

Selvarangan Ponnazhagan, Ph.D.

Department of Pathology, LHRB 513

701, 19th Street South

University of Alabama at Birmingham

Birmingham, AL 35294-0007

Phone: (205) 934-6731

Fax: (205) 975-9927

E-mail: sponnazh@path.uab.edu

INTRODUCTION

Ovarian cancer is the second most common gynecological malignancies in women (1). Because ovarian carcinoma frequently remains clinically silent, a majority of patients with the disease have advanced intraperitoneal dissemination during diagnosis. The mean survival rate for disseminated ovarian cancer is less than 5 years (2, 3). Despite a better understanding of the pathology of the disease, surgery and chemotherapy remain the major therapeutic interventions. Like most of the solid tumors, ovarian cancer growth and metastasis is dependent on new blood vessel formation by the process of angiogenesis (4, 5). Thus, therapies targeting angiogenesis are promising for the control of tumor growth in patients of ovarian cancer. Since the tumoristatic anti-angiogenic therapy targets endothelial cells, effects of this therapy should be sustained without toxicity. Gene therapy approaches appear promising for this purpose. We recently demonstrated using a recombinant adeno-associated virus (rAAV) that stable systemic expression of anti-angiogenic factors following intramuscular vector administration results in significant inhibition of a human epithelial ovarian cancer cell line, SKOV3.ip1, grown as a subcutaneous xenograft in nude mice (6). Although these studies demonstrated the effects of rAAV anti-angiogenic gene therapy, unlike the growth of subcutaneous tumors, the growth of epithelial ovarian cancer is accompanied by excessive intraperitoneal ascites and exfoliation of tumor cells in the peritoneal cavity, which limits the efficacy of drugs and other therapeutic molecules from reaching tumor cells. The presence of ascites at the time of laparotomy for ovarian cancer has also been associated with poor prognosis (7) and the amount of vascular endothelial growth factor (VEGF) in ascites correlates to the disease pathology (8, 9). Thus, in the present study, we sought to determine if rAAV-mediated expression of angiostatin and endostatin as secretory factors following intramuscular administration of the vector will reduce

MATERIALS AND METHODS.

Cells and Reagents. Human embryonic kidney cell line 293 was purchased from American Type Culture Collection and maintained in Iscove's modified essential medium supplemented with 10% newborn calf serum. The human epithelial ovarian cancer cell line SKOV3.ip1 was a gift from Dr. Janet Price and was maintained in Eagle's Minimal Essential Medium containing non-essential amino acids and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Restriction endonucelases and other modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Promega Corporation (Madison, WI). A mouse monoclonal antibody for Ki67 (clone # SP6) and a rabbit polyclonal antibody for PARP p85 fragment were obtained from Research Diagnostics Inc. and Promega, Madison, WI, respectively. Secondary antibodies and color reagents were purchased from Amersham, Piscataway, NJ. Mouse VEGF ELISA kit was purchased from R&D System Inc., Minneapolis, MN.

Recombinant plasmids, production and purification of rAAV. Construction of recombinant plasmids containing secretable form of human angiostatin and endostatin as bicistronically-expressed proteins and that encoding green fluorescence protein (GFP) was recently published (6). Production and purification of rAAV was done by transient transfection in 293 cells followed by iodixanol gradient centrifugation and heparin affinity column chromatography (10). The particle titer of purified virions was determined by quantitative slot blot analysis (11).

***In vivo* studies.** Six-week old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD). All the animal studies

antibody complex was visualized with diaminobenzidine tetrahydrochloride, and tissues were counterstained minimally with hematoxylin.

ELISA. Ascites was harvested on the day of sacrifice of animals due to tumor burden and the volume measured. The ascites fluid was briefly centrifuged to remove loose cells and the supernatant frozen at -80°C until analysis. The VEGF level in ascites was determined using a commercial ELISA kit (R&D System Inc.), which recognized both the 164 and 120 amino acid residues of mouse VEGF.

Statistical Analysis. Data were compiled as mean ± SE in quantitative experiments. For statistical analysis of differences between the groups, an unpaired Student's *t* test was performed. *P* values <0.05 were considered to indicate significant difference between data sets.

with SKOV3 cells (14). Compared to protein or pharmacotherapies, gene transfer approach provides greater benefit for stable systemic levels of the anti-angiogenic factors. The advantages of using rAAV over other vectors are non-pathogenicity, long-term transgene expression and low immunogenicity (15, 16). Our studies established that a single intramuscular administration of rAAV encoding angiostatin and endostatin results in systemic levels of these factors between 177-277 and 176-206 ng/ml respectively in serum after three weeks and remained stable for over 4 months without any apparent toxicity.

The anti-angiogenic mechanism of endostatin and angiostatin are beginning to be discovered. While angiostatin appears to exert anti-angiogenic effect by primarily inhibiting the proliferation and invasion of endothelial cells (17, 18) and inducing endothelial cell apoptosis (19), endostatin reduces endothelial cell proliferation (20) and migration (21), and significantly reduces the invasion of endothelial as well as tumor cells into the reconstituted basement membrane (22). Thus, a combination of angiostatin and endostatin is likely to have increased the inhibitory effect on ascites formation observed in the present studies.

The effects of angiostatin and endostatin on tumor cell proliferation and apoptosis are illustrated in Figure 2. Whereas more than 30% of implanted tumor cells in the rAAV-treated group were apoptotic compared to animals in the naïve group, no significant difference was found between the naïve and rAAV-GFP treated animals ($p>0.05$) indicating the specificity of the transgenic factors. The median proliferation index of tumor cells in rAAV-endostatin + angiostatin treated mice was 22%, significantly lesser than in control group, which showed 61% (Figure 2). Tumor-free animals were monitored up to 150 days before terminating the experiment. The results of the *in vivo* studies are shown in Fig 3. As assessed by tumor-free

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Figure 1

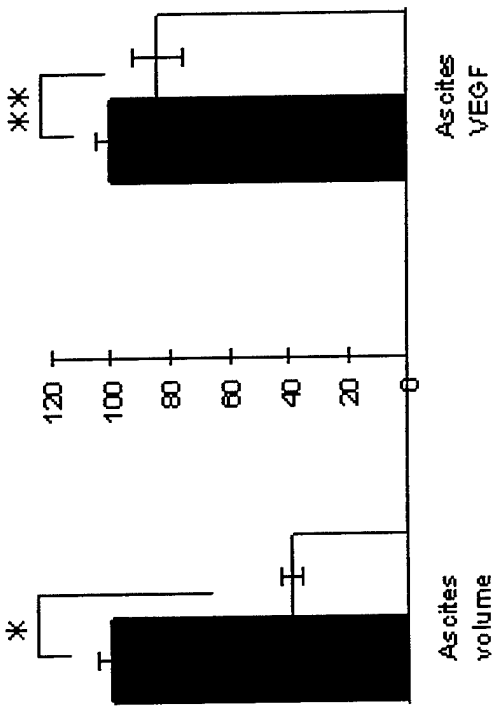
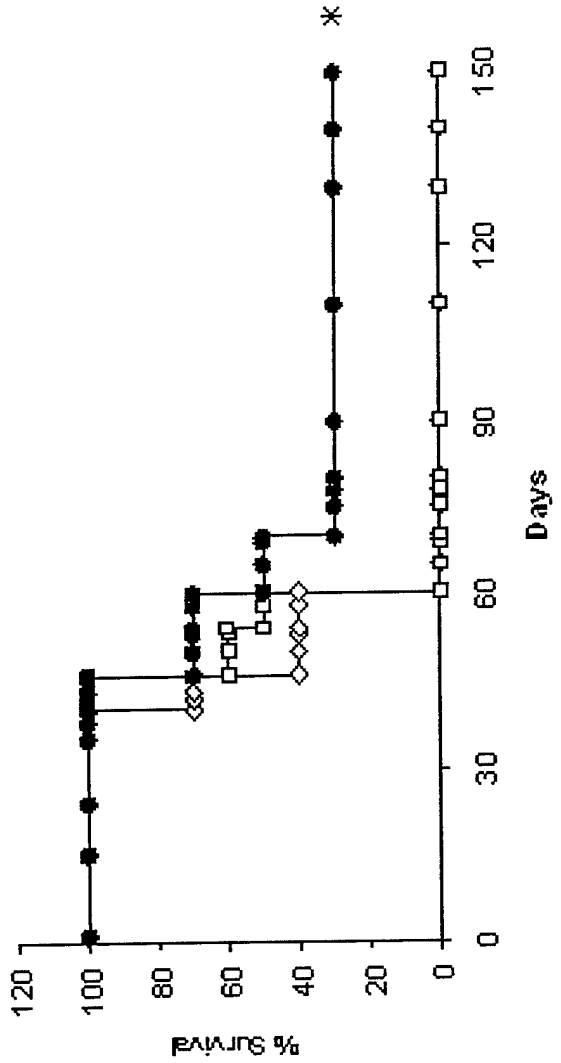
Figure 3